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Role of the AP-1 element and redox factor-1 (Ref-1) in mediating transcriptional induction of DT-diaphorase gene expression by oltipraz: a target for chemoprevention

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Abstract

The dithiolethione oltipraz is a potent chemopreventive agent in preclinical models, and induces the expression of protective enzymes in the colon mucosa and peripheral mononuclear cells of treated human subjects. We investigated the effects of oltipraz on DT-diaphorase expression in HT29 colon adenocarcinoma cells. Following a 24-hr exposure to 100 μM oltipraz, elevated steady-state levels of mRNA for Jun and Fos family members were observed. A nuclear run-on assay showed induction of c-fos and c-jun transcripts at the end of the exposure, peaking at 12 hr after resuspension of cells in drug-free medium. Gel mobility shift analysis revealed a similar time-course of induced nuclear factor binding to an AP-1 probe. Supershift analysis verified the participation of Jun and Fos in the complexes. The redox coactivator Ref-1, a function of which is to enhance AP-1 binding, was induced 5-fold by oltipraz. Immunodepletion of Ref-1 partially inhibited factor binding to the AP-1 probe. Deletion analysis of the DT-diaphorase promoter in a CAT reporter construct revealed that loss of the AP-1 site accounted for approximately 65% of the induction by oltipraz. Mutation of the AP-1 element in a full-length promoter construct yielded similar results. These data suggest the importance of transcriptional activation mediated by AP-1 in the chemopreventive activity of oltipraz, and indicate that novel chemoprevention structures may be selected based upon agonist activity at this locus.

Keywords: Oltipraz; AP-1; c-jun; DT-diaphorase; Ref-1; Chemoprevention

1. Introduction

The prevention of cancer is an urgent and promising direction of biological research. The successful use of retinoids to prevent the emergence of second primary tumors in patients with aerodigestive malignancies indicates that pharmacological approaches may contribute to a decline in cancer incidence in susceptible populations. Dietary constituents associated with a lowered incidence of cancer in many studies include members of the family of Cruciferae and of the genus *Brassicae*, some examples of which are broccoli, brussels sprouts, cauliflower, and cabbage. These vegetables are characterized by high levels of dithiolethiones, isothiocyanates, and indoles, all of which have been demonstrated in preclinical *in vivo* models to have chemopreventive activity. A synthetic dithiolethione, oltipraz [4-methyl-5-(2-pyrazinyl)-1,2-dithiol-3-thione], has been developed for this purpose in preclinical and clinical trials [1]. As the lead compound of its class, a detailed understanding of its protective mechanism is of some importance, both to allow optimization of dithiolethione structures and to permit the development of related and unrelated compounds directed to the same mechanism.

Oltipraz has been shown to inhibit the development of forestomach and pulmonary neoplasia induced by several structurally unrelated carcinogens in mice [2]. In rats, the development of neoplastic liver lesions induced by exposure to aflatoxin B_1 (AFB_1) and the extent of AFB_1–DNA binding *in vivo* are also inhibited by oltipraz [3]. Oltipraz also protects against the acute hepatotoxicity of acetaminophen and carbon tetrachloride. Administration of oltipraz to mice reduces mortality, prevents hepatic glutathione

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Abbreviations: AP-1, activator protein-1; ARE, antioxidant response element; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's minimal essential medium; EMSA, electrophoretic mobility shift assay; GSH, glutathione; NF-κB, nuclear factor-κB; Ref-1, redox factor-1; SSC, 0.3 M NaCl, 0.03 M sodium citrate, pH 7.0; and XRE, xenobiotic response element.

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depletion, and decreases liver damage in animals treated with either toxin [4]. Clapper *et al.* [5] demonstrated that oltipraz protects hamsters from the development of pancreatic cancer following administration of *N*-nitrobis (2-oxopropyl) amine (BOP) [5]. Oltipraz also protects from the colon carcinogenic effects of azoxymethane [6], and Reddy and colleagues have elucidated some of the pharmacologic determinants of drug action [7].

Single doses or chronic administration of oltipraz to mice and rats increases the activities in liver and lung of GSH transferases, GSH reductase, quinone reductase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase [8]. It has been proposed that oltipraz functions by elevating the activities of the Phase II detoxicating enzymes (including DT-diaphorase), primarily through the induction of transcriptional activity [9,10]. DT-diaphorase is one of the Phase II enzymes, coordinate induction of which occurs in response to a wide variety of structurally dissimilar xenobiotics [11,12]. Overexpression of DT-diaphorase has also been observed in human tumor cells resistant to anticancer quinones [13]. Two elements in the 5′ region of DT-diaphorase have been shown to mediate transcriptional induction. The XRE is required for transcriptional activation in response to planar aromatic compounds, azo dyes, dioxin, and other polycyclic xenobiotics [14]. The XRE is also found in the promoter region of the cytochrome P450 1A1 gene [15]. The mechanism through which the XRE stimulates transcription is known for compounds that interact with the Ah receptor: translocation of the ligand-bound receptor from the cytosol to the nucleus, followed by binding to the XRE, results in transcriptional activation [16]. Other proteins that bind to this element have not been identified, but could conceivably mediate the actions of xenobiotics acting through alternative mechanisms.

The second response element is the ARE, which is also found in the 5′ region of the rat GSH transferase Ya gene [17]. The ARE was originally described as a sequence of about 40 bp conserved among a variety of enzymes responsive to various monofunctional inducers [18,19]. Binding of AP-1 proteins to the ARE in response to electrophiles has been demonstrated [20,21], but the functional relevance of this binding remains controversial [21,22]. Comprehensive mutational analysis of the murine GSH transferase Ya ARE has determined that transcriptional induction by *tert*-butylhydroquinone depends upon substantial homology with a 16-bp segment. In this model, induction of γ-glutamylcysteine synthetase, the promoter of which also contains an ARE, was not observed. With oltipraz in human cells, however, strong induction of both DT-diaphorase and γ-glutamylcysteine synthetase was found [21]. Therefore, the participation of AP-1 proteins acting through this element in oltipraz-treated cells was investigated.

We have shown that transcriptional induction of detoxication genes through AP-1 may account for increased detoxicating enzyme activity under hypoxic conditions [23]. To determine the molecular basis for chemoprevention by oltipraz through AP-1, we defined the relevant *cis*-acting elements using deletion mutants and mutational analysis of the DT-diaphorase promoter. Additional proteins capable of participating in promoter binding with AP-1 were investigated. One of these, Ref-1, subserves a reducing function to maintain Jun and Fos proteins in a conformation that promotes AP-1 binding. We have demonstrated previously [24] the participation of this protein in a model of drug resistance, and in this study we investigated its potential role in the mechanism of oltipraz.

2. Materials and methods

2.1. Cell culture and treatment

HT29 colon adenocarcinoma cells were grown in DMEM with 10% fetal bovine serum in 75 cm^2 flasks at 37° in a humidified atmosphere of 95% air/5% CO_2. Cells were seeded at a density of 5×10^6/flask, allowed to adhere overnight, and treated with 100 μM oltipraz (which was obtained from the Division of Cancer Prevention and Control, National Cancer Institute) for 24 hr at 37°. After the oltipraz was removed, the cells were maintained in fresh medium and harvested at intervals after treatment.

2.2. Northern blotting

Total RNA was isolated using the TRIzol reagent (Gibco BRL) according to the instructions of the manufacturer. Briefly, 10^7 cells were lysed in 3 mL of TRIzol reagent and incubated for 5 min at room temperature. After adding 0.6 mL of chloroform, the tube was vortexed and incubated at room temperature for 5 min. The sample was centrifuged at 12,000 *g* for 15 min at 4°. The aqueous phase was saved, and the RNA was precipitated by mixing the aqueous phase with 1.5 mL isopropyl alcohol, and centrifuging at 12,000 *g* for 15 min at 4°. The RNA pellet was washed once with 70% ethanol, and dissolved in diethylpyrocarbonate (DEPC)-treated water. RNA was electrophoresed in a 1% agarose denaturing gel. The RNA was transferred to nylon membranes, and hybridized to probes as follows: (a) a 1.2 kb human DT-diaphorase probe [25], (b) the 1.8 kb *Bam*HI-*Eco*RI insert of a human c-*jun* [26], (c) the 3.1 kb *Xho*I-*Nco*I fragment of a human c-*fos* [27], (d) the 1.0 kb *Xho*I fragment of a human *jun*-B [28], (e) the 0.8 kb *Xho*I fragment of a human *jun*-D [28], (f) the 1.4 kb *Eco*RI insert of a human *ref-1* [29], and (g) β-*actin*. Membranes were washed for 1 hr in 2× SSC containing 0.5% SDS and at 60° followed by 1 hr in 0.1× SSC containing 0.5% SDS. The filters were exposed to X-ray film at −70° for 1–5 days.

2.3. Nuclear run-on assay

Nuclei from oltipraz-treated and control cells were prepared as described previously [30]. The cells were

washed with ice-cold PBS and scraped into buffer A [20 mM Tris–HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$] following which they were made 0.1% by volume with Nonidet P-40. The cells were vortexed, and the plasma membrane was lysed in sterile conditions at 1000 *g* for 10 min at 4°, washed in cold buffer A, and counted. The nuclear pellet was resuspended in transcription buffer [35% glycerol, 10 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 80 mM KCl, 0.1 mM EDTA] and stored at −70°. Following thawing, the run-on assay was conducted with 10^8 nuclei per reaction mixture in a total volume of 200 μL in transcription buffer with a 4 mM concentration (each) of ATP, GTP, and CTP and 200 μL of [α-^{32}P]UTP (3000 Ci/mmol; Amersham) at 26° for 10 min. Nuclei were digested with 10 μL of RNase-free DNase I, 10 μL of 10× SET [5% SDS, 50 mM EDTA, 10 mM Tris–HCl (pH 7.4)], and 5 μL of yeast tRNA (10 mg/mL) at 37° for 30 min. Nuclear RNA was isolated by the TRIzol reagent following the instructions of the manufacturer (Gibco BRL). Finally, the RNA was dissolved in Tris–EDTA with 0.1% SDS solution. The DNA probes DT-D, c-jun, c-fos, and β-actin (2 μg of DNA per blot) used in the run-on assay were denatured and blotted onto a pre-wet nylon slot filter membrane in 6× SSC and allowed to dry at room temperature. The membrane was baked at 80° for 2 hr in a vacuum oven. After prehybridization of the membrane at 42° for several hours, the [α-^{32}P]-labeled nuclear RNA in 3 mL of hybridization buffer was added to the 2× SSC–1% SDS at 65° for 1 hr, and then in 0.1× SSC–0.1% SDS at room temperature for 1 hr. Autoradiography was performed at −70°, and quantitation of the results was achieved by densitometric scanning normalized to the signal for β-actin.

2.4. EMSA

The nuclear extracts were prepared by a modification of the procedure of Dignam *et al*. [31]. Cells were harvested and resuspended in 1.5 vol. of lysis buffer containing 25 mM HEPES (pH 7.5), 70 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM sodium orthovanadate, 0.5 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, and 1.0 mM dithiothreitol. The mixture was incubated on ice for 20 min and then extracted by adding 1.6 vol. of extraction buffer containing 25 mM HEPES (pH 7.5), 0.5 mM EDTA, 20% glycerol, 1.66 M KCl, 0.4 mM NaF, 0.4 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, and 1.0 mM dithiothreitol with constant shaking at 4° for 4–6 hr. Samples were centrifuged at 55,000 *g* for 1 hr at 4°. The supernatant was dialyzed at 4° for 4–6 hr in 20 mM HEPES (pH 7.5), 50 mM KCl, 0.1 mM EDTA, 10% glycerol, 0.4 mM NaF, 0.4 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, and 1.0 mM dithiothreitol. Samples were stored at −70°. Protein content was assayed by the Bradford method (Bio-Rad).

The nuclear extracts were analyzed for AP-1-binding activity by EMSA. The binding reaction mixture contained 10 μg of nuclear extract and 1.8 μg of poly(dI-dC) in a 30 μL final volume of binding buffer containing 20 mM HEPES (pH 7.5), 400 mM KCl, 1.0 mM $MgCl_2$, 0.1 mM EGTA, and 0.5 mM dithiothreitol. Each reaction mixture contained 15,000 cpm of a 31-bp double-stranded AP-1 consensus oligonucleotide. The 31-bp DT-diaphorase AP-1 consensus and mutant oligonucleotides shown previously [23] were synthesized. cDNA standards were purified and annealed by standard procedures [32]. The double-stranded oligonucleotides were labeled with [γ-^{32}P]ATP by phosphorylation with bacteriophage T4 polynucleotide kinase and then ethanol precipitated to remove the bulk of the unincorporated radioactivity. The EMSA reaction mixture was allowed to stand for 25 min at room temperature. After the addition of 5 μL of loading buffer (20% Ficoll, 0.25% bromophenol blue), 15-μL samples were loaded onto a 4% polyacrylamide gel and electrophoresed at 4° for 2 hr at 200 V. The gel was dried under vacuum and exposed to X-ray film overnight at −70°.

For the supershift assay, the nuclear extracts were pre-incubated with anti-c-Jun or anti-c-Fos (Santa Cruz Biotechnology Inc.) before analysis by EMSA as described above.

2.5. Western blotting

The nuclear extract proteins (15 μg/lane) were loaded on SDS–12% polyacrylamide gels, electrophoresed, and transblotted to a Hybond-P membrane (Amersham). Western blotting was carried out using the Ref-1 rabbit antiserum as the first antibody and horseradish peroxidase-conjugated serum as the second (Santa Cruz Biotechnology Inc.), and the ECL-Plus detection system (Amersham).

2.6. Immunodepletion assay

Immunodepletion was conducted using a standard procedure [33]. The nuclear extract was incubated with 1:10 volume of Ref-1 rabbit antiserum at 4° for 1 hr with gentle shaking. After the immunocomplexes were cleaned by immunoprecipitation, the antibody–antigen complex was used for SDS–PAGE and subsequent Coomassie blue stain analysis. The cleaned supernatant was used in EMSA for the stimulation of AP-1 DNA-binding activity as described above.

2.7. CAT assay

The structure of the DT-diaphorase promoter has been described previously [34]. The mutant constructs were synthesized by the Ex-site PCR-based site-directed mutagenesis method following the procedure of the manufacturer (Stratagene). The mutation site in AP-1 was the same site as used in the EMSA described above.

HT29 cells were plated at 1×10^6/100-mm dish 24 hr before transfection. Transfections were performed with

20 μg of plasmid construct DNA using the calcium phosphate precipitation method (5′ Prime-3′ Prime, Inc.). After an additional 24-hr incubation, cells were harvested, washed, and pelleted. Finally, the pellet was resuspended in 200 μL of 0.25 M Tris buffer, pH 7.8, containing 1.0 mM phenylmethylsulfonyl fluoride per 100-mm dish and frozen at −70°. The lysates were obtained by three freeze/thaw cycles. The protein concentration was determined by the Bradford assay. A total of 50 μg of total cellular protein was assayed by an enzyme-linked immunosorbent assay-CAT method (5′ Prime-3′ Prime, Inc.). Transfection efficiency, as determined by cotransfection with a β-gal plasmid (CMV-β-gal), was highly uniform within the HT29 cell line. The CAT activities were expressed as activity relative to that of the pCAT basic control construct. The reported values are the means of three different transfections, each performed in duplicate.

3. Results

3.1. Induction of AP-1 transcription factors

Previous experiments defined the optimal concentrations and exposure time for oltipraz treatment of HT29 cells (100 μM for 24 hr). DT-diaphorase steady-state mRNA levels were unchanged at the end of the 24-hr exposure, and increased by about 8-fold over the subsequent 24 hr (Fig. 1). The AP-1 element is a component of the ARE that regulates DT-diaphorase expression in response to antioxidants. We performed northern analysis to determine the RNA expression of AP-1 constituents. Among the Jun family transcription factors, c-jun was the most markedly induced (7-fold), while the content of jun-B and jun-D was increased only about 2-fold. All showed a peak at the end of the 24-hr exposure to oltipraz. The elevated expression of c-jun was maintained for 12 hr, by which time jun-B and jun-D had returned to baseline. Expression of c-fos was also induced by oltipraz and increased 4-fold; the elevation was sustained for 12 hr. These data show that c-jun and c-fos have the greatest degree of response to oltipraz treatment.

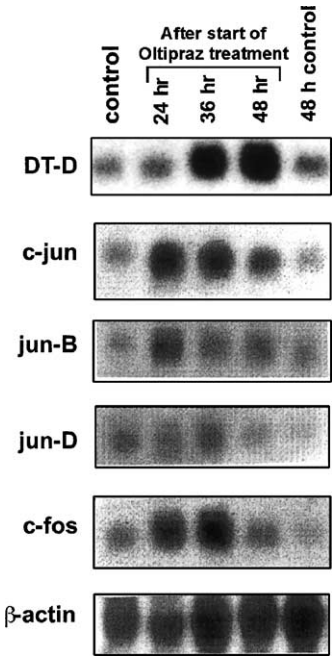

Fig. 1. Northern analysis of the expression of transcriptional factors relevant to AP-1 in HT29 cells after exposure to oltipraz for 24 hr. Total RNA was blotted and probed for DT-diaphorase (DT-D) and for AP-1 constituent transcription factors as noted. A β-actin probe was used as a loading control.

To verify that transcriptional induction is the major mechanism of oltipraz action through AP-1, we performed a nuclear run-on analysis (Fig. 2). Consistent with the steady-state mRNA data, a marked increase in the rate of c-jun and c-fos transcription was observed at the end of the 24-hr exposure to oltipraz, at which time the DT-diaphorase expression rate was unchanged. The transcriptional rate of c-fos and c-jun peaked at 12 hr, and returned to baseline by 24 hr. A later peak of DT-diaphorase induction was observed at 24 hr after the end of the exposure. The temporal characteristics of these responses suggest a causal relationship.

Verification of similar responses at the protein level was sought by EMSA (Fig. 3A). Incubation of nuclear protein isolated with a labeled authentic DT-diaphorase AP-1 31 bp oligonucleotide at various times after exposure to oltipraz revealed the induction of AP-1-binding transcrip-

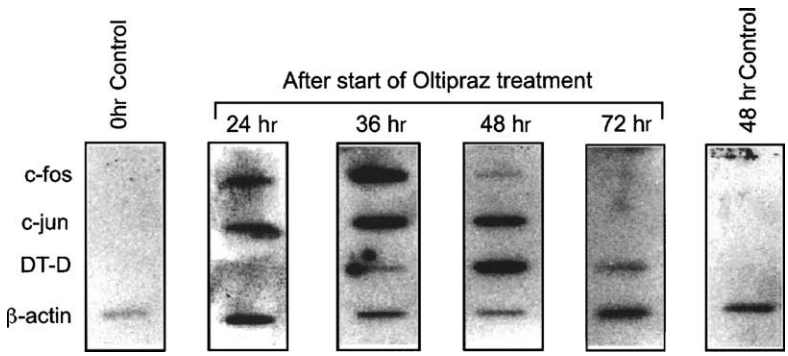

Fig. 2. Nuclear run-on assay of c-fos, c-jun, and DT-diaphorase RNA in oltipraz-treated and untreated human HT29 cells. Probes were immobilized on a nylon membrane and hybridized to RNA derived from 10^8 nuclei per reaction. β-Actin was used as a control.

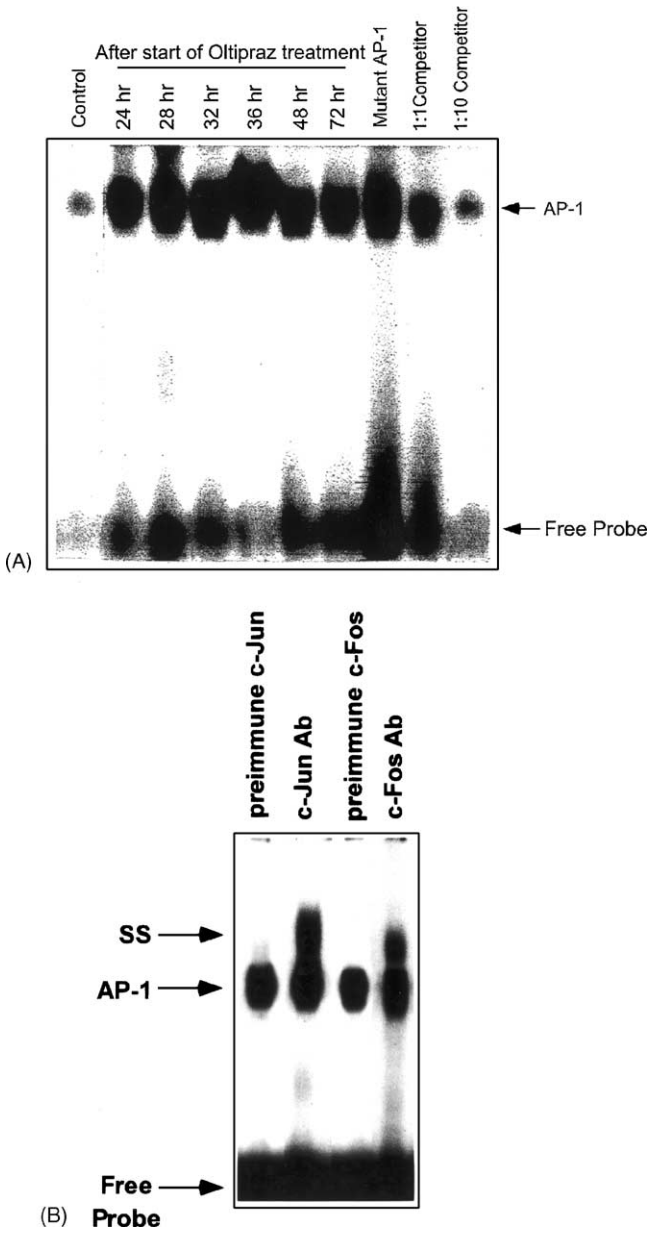

Fig. 3. (A) EMSA of AP-1-binding activity in protein extracts from cultured human HT29 cells exposed to oltipraz. A [^{32}P]-labeled synthetic double-stranded oligonucleotide containing consensus DT-diaphorase AP-1 was used as the probe. The oltipraz-inducible DNA–protein complexes formed after expression of the cells to oltipraz are indicated (arrow). The unbound (free) probe in the gel is indicated at the bottom. Oltipraz-inducible DNA-binding activity was partially abolished by competition with a 1:1 and 1:10 fold molar excess of unlabeled natural AP-1 oligonucleotide (competitor lanes) but was not affected by an identical concentration of oligonucleotide containing a mutant DT-diaphorase AP-1 (mutant lane). (B) Supershift assay of DT-diaphorase AP-1-binding activity in HT29 nuclear extracts of oltipraz-treated HT29 cells harvested at 12 hr. Preincubation of nuclear extract (10 μg) from HT29 cells treated with oltipraz and anti-c-Fos and anti-c-Jun before EMSA resulted in a supershifted band consistent with the formation of DNA–protein–antibody complexes.

tion factors. The protein–oligonucleotide complex formation followed a time-course similar to that for transcription factor RNA induction. There was no detectable time-lag for protein binding, as might be expected from this sparse

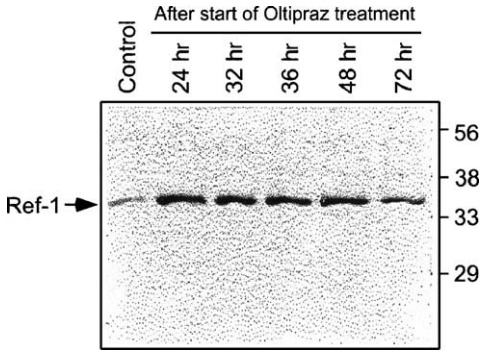

Fig. 4. Western blot analysis of human Ref-1 protein in HT29 cells exposed to 100 μM oltipraz for 24 hr. Molecular markers are shown on the right side of the figure.

sampling schedule. As with c-jun and c-fos mRNA levels, peak probe binding occurred 12 hr after the end of exposure, with subsequent diminution of effect. Interestingly, evidence for sustained elevated protein binding to the AP-1 probe was obtained at 48 hr, when jun and fos mRNA expression had returned to baseline values.

We hypothesized that Fos and Jun family proteins may be responsible for the early response to oltipraz, but that additional factors might underlie its maintenance. To verify this, we performed supershift analyses using anti-c-Jun and anti-c-Fos antibodies over the entire time-course from initial exposure through the 48-hr point. These studies revealed that c-Jun and c-Fos containing complexes were present at 12 hr (Fig. 3B), but that the AP-1 probe shifting activity was possibly mediated by other transcription factors at the later time points.

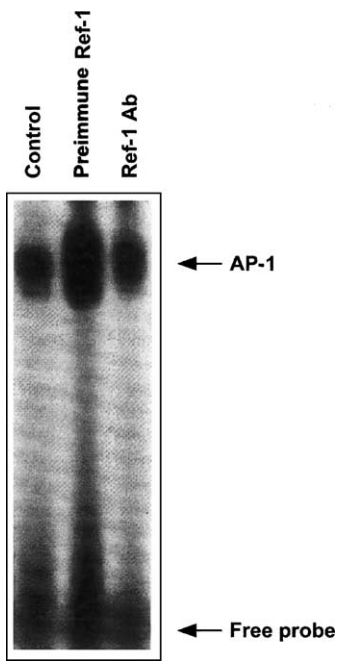

Fig. 5. Effects of immunodepletion of Ref-1 on AP-1-binding activity in the nuclear extract of HT29 cells treated for 24 hr with oltipraz and cultured in drug-free medium for 12 hr.

3.2. Activation of Ref-1 by oltipraz

The bifunctional nuclear protein Ref-1 was found by Xanthoudakis *et al.* [29] to have a redox function, and by Hickson and colleagues to be an endonuclease [35]. The former role is critical to maintaining a reduced configuration of cysteines in the vicinity of the DNA-binding motifs of several transcription factors, including Jun and Fos. Loss of Ref-1 function greatly diminishes the affinity of the transcription factors for their cognate DNA elements. The western blot (Fig. 4) shows that oltipraz treatment induced Ref-1 protein content by about 5-fold, and that the effect was maintained up to 48 hr. The functional importance of this observation was explored by immunodepletion experiments in an EMSA assay (Fig. 5). A 12-hr oltipraz-treated nuclear preparation incubated with pre-immune serum showed the

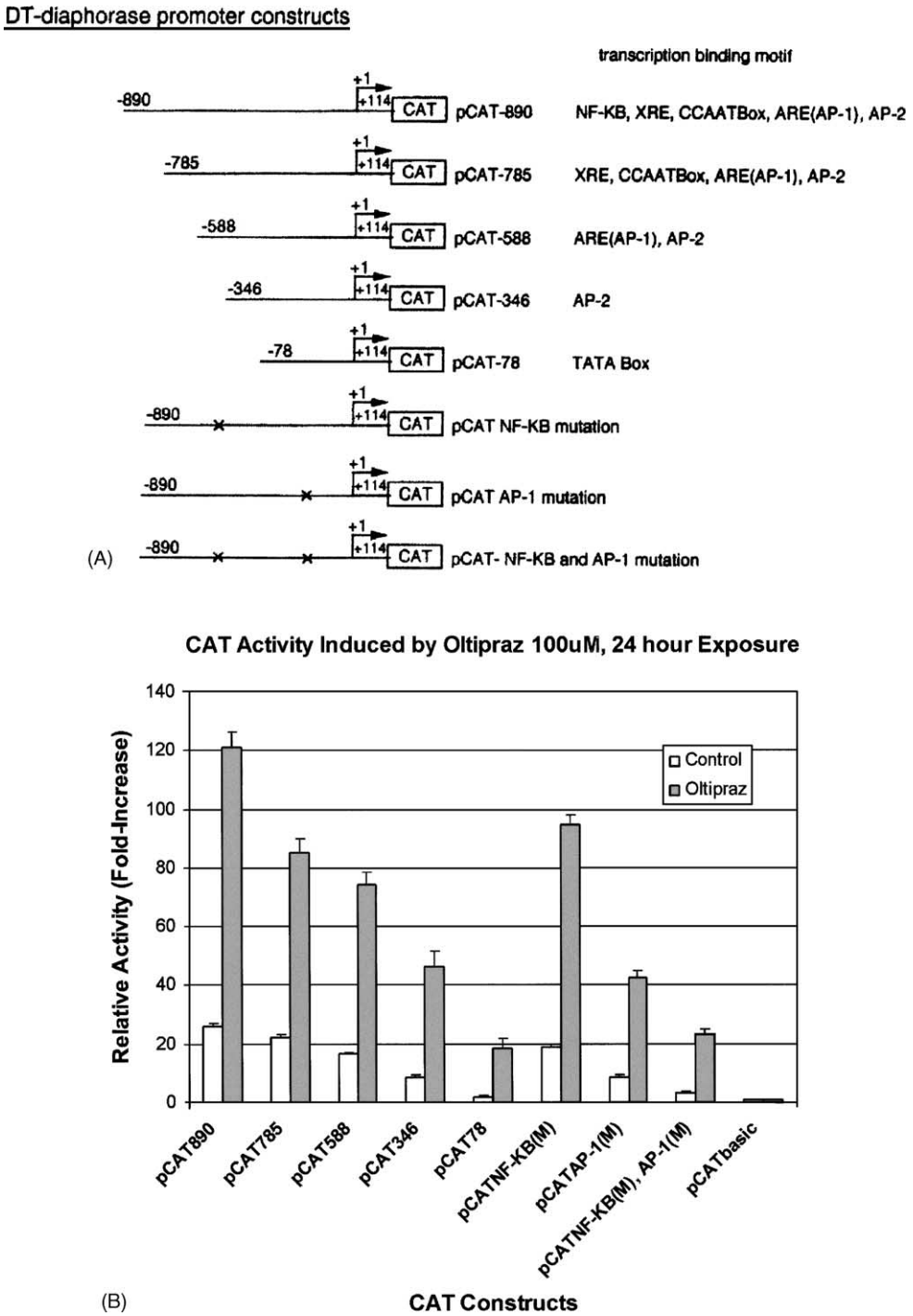

Fig. 6. (A) Construction of various DT-diaphorase gene 5′-flanking regions in CAT vector. (B) Effects of oltipraz on the activities of CAT constructs containing various lengths of DT-diaphorase gene 5′-flanking regions in HT29 cells. CAT activity of each construct was calculated as activity relative to that of the CATbasic control construct. The reported values are the means of three different transfections, each performed in duplicate (mean ± SD).

expected pronounced shift of the AP-1 probe. When the nuclear extract was incubated with an excess of anti-Ref-1 antibody, depletion of factor binding to the probe was demonstrated. Control binding was unaffected by Ref-1 immunodepletion (data not shown) consistent with the known role of Ref-1 in response to altered oxidation status. Thus, the elevation in Ref-1 content plays a critical role in the binding of induced transcription factors to AP-1.

3.3. DT-diaphorase promoter analysis

We have previously described DT-diaphorase deletion mutants in a CAT reporter construct. The constructs (Fig. 6A) were used to define the *cis* elements responsible for the transcriptional induction of DT-diaphorase following mitomycin C treatment of HT29 cells [34]. We used site-directed mutagenesis to mutate specifically the AP-1 and NF-κB elements, or both. HT29 cells transiently transfected with these constructs were treated with 100 μM oltipraz for 24 hr and harvested at 12 hr. CAT activity was quantitated by the CAT/enzyme-linked immunosorbent assay, and the results are shown in Fig. 6B. The transfection with plasmid pCAT-890 expressed the *CAT* gene at a high level following oltipraz treatment. Activity 120.9- and 25.9-fold over basic CAT control activity (2.4 ng CAT protein/mg total protein) was observed in oltipraz-treated and control cells, respectively. Thus, there was a 4.7-fold induction of DT-diaphorase transcription with oltipraz treatment at the optimal concentrations (Fig. 6B). The difference between pCAT-890 and pCAT-785 (29.6%) represents the contribution of binding to NF-κB, an element we have shown previously to be involved in the oltipraz response [24]. A small decrement (8.9%) was observed between pCAT-785 and pCAT-588, a region that contains a CCAAT box. Between pCAT-588 and pCAT-346, a region that contains the AP-1 site in an ARE, approximately half the remaining activity was lost, or 23.1% of the total (relative to pCAT-890). Additional elements contribute a small amount of the total in the region between AP-1 and the transcription start site. To determine the specificity of these deletions for the NF-κB- and AP-1-binding elements, we performed site-specific mutagenesis of the pCAT-890 construct as described (Fig. 6A). The specifically mutated constructs confirm these findings: the NF-κB mutant lost 21.6% of transcriptional activity while the AP-1 mutant lost 64.8%, somewhat more than expected. The double mutant lost 81.3% of the activity of the full-length construct. These data support the key role of AP-1 in mediating the transcriptional response to oltipraz.

4. Discussion

The development of neoplasia depends on DNA damage from exogenous and endogenous mutagens, and the capacity of the cell to neutralize their effect or consequences. The role of environmental mutagens in causing lung, bladder, and colon cancers is well-recognized. Susceptibility to cancer is increased in individuals with diminished capacity to detoxicate mutagens, as evidenced by over-representation of particular glutathione transferase allelotypes in patients with lung cancer [36]. Emerging data associate cancer risk with cytochrome P450 oxidoreductase profiles that favor mutagen persistence [37]. One may reasonably hypothesize, therefore, that maximizing detoxication potential could diminish the cellular effects of mutagens, and so contribute to cancer prevention.

This hypothesized mechanism underlies the development of oltipraz, a dithiolethione with chemopreventive activity in preclinical models [2]. Oltipraz has been shown to increase the activity of Phase II enzymes and to promote the metabolism of carcinogens in murine models [8]. Kensler's group has shown that oltipraz increases aflatoxin metabolism in treated human subjects in a high-risk population [3]. We previously demonstrated that the induction of DT-diaphorase in HT29 cells by oltipraz is mediated through transcriptional induction, and that the NF-κB element in the DT-diaphorase promoter is functional in this context [24]. In the present work, we report a full characterization of the effects of oltipraz on DT-diaphorase promoter function.

Previous studies of this promoter by Jaiswal have elucidated several regions that contribute to basal and induced promoter activity [38]. Prominent among these are elements common to several detoxication genes, the XRE and ARE. The latter contains within it an AP-1 element, the function of which has been questioned. As shown in Figs. 1 and 2, induction of AP-1-binding proteins of the Jun and Fos families follows exposure to oltipraz. EMSA analysis demonstrated increased nuclear content of AP-1-binding proteins. These findings suggest a role for Jun and Fos proteins in the inductive response. However, the EMSA time-course, with persistent shifts at time points at which Jun and Fos have decreased, suggests the participation of additional factors. Venugopal and Jaiswal [39] have described two transcription factors, Nrf-1 and Nrf-2, members of the basic-leucine zipper family of transcription factors, that bind ARE, and are functional in hepatoma cells. Ramos-Gomez *et al.* [40] have shown that induction of DT-diaphorase in the liver and forestomach of oltipraz-treated *Nrf-2* knockout mice was attenuated markedly compared to the wild-type control. These data suggest that factors additional to Jun and Fos proteins acting on the ARE may be critical to the effects of oltipraz. The requirement for Jun and Fos to interact with Nrf-2 has not yet been defined, and may be relevant to future chemoprevention target identification.

The relevance of transcriptional activation through the AP-1 element is addressed by the deletion and mutagenesis studies presented here. A role for AP-1 is demonstrated and quantified: in this model, 23% of the DT-diaphorase promoter activity is accounted for by sequences containing

AP-1. This activity is confirmed (64.8%) by mutation analysis of the specific AP-1-binding sequences. The disparity between these two values is not explained, but it is consistent with a loss of both AP-1 activity *per se* together with its known cooperativity with NF-κB [24], a factor that would have been eliminated in the initial deletion mutant (Fig. 6B). Taken together with specific super shifting of the EMSA complex with c-Jun and c-Fos antibodies, the data argue strongly for participation of AP-1 proteins in the regulatory complex. How the Nrf-1 and -2 factors interact at this locus is unclear, but it likely involves interactions between these factors.

The affinity for AP-1-binding proteins for the DNA element is dependent upon a reduced state of cysteines close to the DNA-binding motif [29]. These cysteines are the target of a bifunctional nuclear protein Ref-1, which has both reducing and AP-endonuclease functions. We previously indicated that Ref-1 participates in the transcriptional response to hypoxia [23]. Here, we observed induction of Ref-1 by oltipraz. The time-course of Ref-1 induction is parallel to that of AP-1-binding activity as detected on EMSA, and competition with Ref-1-binding antibodies suggests a functional role for this protein in the transcriptional effects of oltipraz. It is unknown if Ref-1 may also interact with Nrf proteins, but this protein modifies the affinity for DNA binding of multiple transcription factors including Pax-5, Pax-8, HIF-1, and p53 [41–44]. Ref-1 also interacts with NF-κB, promoting binding of Rel proteins [45,46]. As noted above, while NF-κB alone seems to account for a substantial proportion of oltipraz-induced promoter activity (22–30%), its coactivator interactions with AP-1 may be of additional importance.

We have focused here on the mechanisms of induction of detoxicating enzymes that are often associated with the chemopreventive activity of oltipraz. Additional mechanisms have been identified, including stimulation of nucleotide excision repair [47], and induction of the activity of ATP-binding cassette transport proteins such as MRP-2 [48]. These additional mechanisms, and possibly others, are plausible candidates for the ultimate mechanism by which oltipraz protects cells from what would be a carcinogenic insult. The transcriptional mechanisms identified here may also have roles to play in each of these settings, and therefore the mechanisms themselves are potential targets for the development of chemoprevention drugs.

Together these data support the role of ARE and its constituent AP-1 site in the oltipraz response. We have found that oltipraz in the clinic is too toxic for extended routine use as a chemoprevention candidate [49]. Talalay's group previously utilized a Phase II enzyme promoter to optimize selection of chemoprevention candidates [50,51]. With the caveat articulated by Ramos-Gomez *et al.* [40] that regulatory controls may be cell-type specific, it is reasonable to propose an ARE-/AP-1 model for both dithiolethione optimization and for discovery of novel structures with chemopreventive activity.

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