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# RAPID COMMUNICATION

## INVOLVEMENT OF NF- κB IN THE INDUCTION OF NAD(P)H:QUINONE OXIDOREDUCTASE (DT-DIAPHORASE) BY HYPOXIA, OLTIPRAZ AND MITOMYCIN C

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**ABSTRACT** - The activity of the two-electron bioreductive enzyme DT-diaphorase (DTD) is induced by heat shock, hypoxic stress, oltipraz, and mitomycin C (MMC). Transcriptional induction is associated with nuclear factor binding to elements mediating immediate early responses including AP-1, though the DTD mRNA peaks at 24 hr. Electrophoretic mobility shift assays revealed that nuclear protein extracts from hypoxia-, oltipraz-, and MMC-treated cells bound a specific oligonucleotide probe corresponding to the NF-κB transcriptional binding site in two human cancer cell lines, HT29 and HepG2. The binding activity for the NF-κB site was induced with a time-course similar to that of the induction of DTD, and was delayed in comparison to the induction of AP-1 binding proteins. The time-courses of the NF-κB binding response to MMC, oltipraz and hypoxic treatment were similar, and binding was most pronounced at 24 hr. All three stimuli were associated with the late appearance of a higher molecular weight complex in HT29 but not in HepG2 cells, suggestive of the participation of additional rel family proteins in DNA binding in this cell line. Competition experiments indicated that the bound protein complex was specific for the NF-κB binding site. An immunodepletion assay showed that in each case the bound complex consisted of a heterodimer of the NF-κB proteins p50 and p65. These data suggest that hypoxia, oltipraz and MMC may each induce the overexpression of DTD through a mechanism involving the NF-κB response element in the DTD 5′-flanking region, and support a role for this element in the control of detoxication responses to environmental changes.

*Key words:* NAD(P)H:quinone oxidoreductase; DT-diaphorase; gene expression; NF-κB; hypoxia; oltipraz; mitomycin C

Bioreductive enzymes are responsible for both the activation and the detoxication of xenobiotics under oxic and hypoxic conditions [1]. The cellular pharmacology of MMC[+] in oxic cells depends on the generation of reactive oxygen intermediates as a result of two-electron reduction of the quinone by DTD [2]. The sensitivity of tumor cell lines to MMC has been related to their DTD activity [2,3]. Under other circumstances, two-electron reduction may protect the cell from toxic agents [4]. One of the effects of the chemoprotective agent oltipraz is the elevation of colon DTD activity *in vitro* and *in vivo*, through a mechanism that appears to involve transcriptional induction [5,6]. The enhanced capacity to metabolize mutagens through the action of this and other detoxication enzymes has been associated with the chemopreventive action of oltipraz. Thus, bioreductive enzymes play a central role in the metabolic transformation of reactive compounds.

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[+] Abbreviations: MMC, mitomycin C; DTD, DT-diaphorase; PCR, polymerase chain reaction PMSF, phenylmethylsulfonyl fluoride; and DTT, dithiothreitol.

Among the best characterized of these enzymes at a molecular level is DTD, the gene for which has been cloned in both rats [7] and humans [8]. In both species, the promoter regions have been sequenced, and particular response elements have been characterized. These include particularly the xenobiotic response element and the antioxidant response element. The former is common to both species and appears to respond to a variety of structures [8-10]. The latter is similar in both species, and is responsive to antioxidants such as β-napthoflavone and phenolic antioxidants [9,11]. The human DTD promoter region, however, contains a consensus AP-1 sequence that is absent in the rat gene [8]. This ubiquitous DNA element, also known as the TRE, binds the transactivating protein families Fos and Jun, as Jun homodimers or Jun-Fos heterodimers [12,13]. We have demonstrated previously the involvement of the AP-1 element in the induction of DTD activity that follows either treatment with MMC [14] or an 8-hr exposure to hypoxic conditions [15,16].

Examination of the sequence of the DTD promoter reveals another element in proximity to the AP-1 binding site. The NF-κB element was identified as a binding site for nuclear transactivating factors in the immunoglobulin light chain enhancer [17]. Subsequent work has shown that target genes for the NF-κB protein include immunomodulatory cytokines, immunologically relevant cell surface receptors, and acute phase response proteins [18]. NF-κB consists of two proteins (p50 and p65), which in unstimulated cells reside in the cytoplasm bound to a specific inhibitor of their DNA binding, I-κB [19]. Upon activation, p50 and p65 dissociate from I-κB, translocate to the nucleus, and bind DNA as a p50 homodimer or a p50-p65 heterodimer [20]. In light of recent data implicating NF-κB in the response to redox processes [21,22], we sought to investigate the possible role of NF-κB in the response of DTD to disparate stimuli that result in its overexpression. We found that MMC, oltipraz and hypoxia all elicited binding to the NF-κB element in the DTD 5′-flanking region, and that the time-course was consistent with that of the induction of DTD mRNA.

## MATERIALS AND METHODS

Cell culture. Human HT29 colon adenocarcinoma and HepG2 hepatoma cells were maintained by growing cells in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum in 75-$cm^2$ flasks or 500-mL milk dilution glass bottles with 1.0 x $10^6$ cells in a humidified atmosphere of 95% air in 5% $CO_2$, and were exposed to hypoxia or drug 2 days later.

Hypoxia, drug treatment. To study the effects of hypoxia, the glass bottles were rendered hypoxic by passage of oxygen-poor 95% $N_2$-5% $CO_2$ over the cells for 2 hr. The bottles were then sealed for a further 6 hr, after which oxic conditions were restored. This treatment resulted in $O_2$ concentrations substantially less than 1%; further evidence of profound hypoxia was provided by a 2 orders of magnitude decrease in the $IC_{50}$ of the hypoxic toxin SR4233 under these conditions (O'Dwyer PJ, unpublished results). As previously noted [15], this duration of hypoxic exposure resulted in only 14% loss of viability by trypan blue exclusion; when total protein values were used, the mean loss of viability at 48-72 hr after treatment was 28%. Drug-treated cells were exposed to 1.25 μM MMC for 4 hr at 37°, or to 100 μM oltipraz (dissolved in DMSO) for 24 hr at 37° after which cells were maintained in fresh medium. This concentration of MMC is an $IC_{90}$ for HT29 cells, whereas oltipraz at this concentration is not cytotoxic (by clonogenic assay). The cells were harvested before and at 24 and 48 hr after treatment.

DTD gene expression. Total cellular RNA was prepared from the cell by the acid guanidinium isothiocyanate phenol-chloroform extraction method described by Chomczynski and Sacchi [23]. An aliquot equivalent to 20 μg of each RNA preparation was subjected to northern blotting. The blot was hybridized with a $^{32}P$-labeled 1.4 kb human DTD cDNA probe. ** After the blot was washed in 1x SSC, 0.1% SDS for 20 min at room temperature, in 0.2x SSC, 0.1% SDS for 20

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min at 55° and in 0.1x SSC, 0.1% SDS for 20 min at 55°, autoradiography was carried out at -70° for 3-7 days (1x SSC is 0.15 M NaCl, 0.01 M $NaH_2PO_4 \cdot H_2O$, 0.001 M EDTA, pH 7.6). The intensity of β-actin labeling was used as an internal normalizing value.

As an additional means of quantitating DTD gene expression, the reverse transcriptase PCR assay was used based on a method previously described [24]. A 100 ng sample of HepG2 total RNA for each time point was used for cDNA production. Further PCR amplication was carried out with 35 cycles at 94° for 1 min with the following DTD PCR primers containing the $T_7$ polymerase promoter sequence attached to their 5′ ends: DTD-54-$T_7$ - "GGAG" AGGCTGGTTTGAGCGAGTGTTC (bases 392-413 of the DTD coding sequence) [25]; and DTD-56 ATTTGAATTCGGGCGTCTGCTG (bases 640-661 of the DTD gene coding sequence) [25]. The β-actin gene primer BA-67 $T_7$-"GGGAGA" GCGGGAAATCGTCGTGCGTGACATT (bases 2104-2127 of the β-actin genomic sequence) [26] and BA-68 GATGGAGTTGAAGGTAGTTTCGTG (bases 2409-2432 of the β-actin genomic sequence) [26] were used for an internal control. For $T_7$ polymerase transcription, 3 μL of PCR product was used with 0.25 μL [γ-$^{32}$P]CTP (3000 Ci/mmol), and 50 units of $T_7$ RNA polymerase solution. The transcription mixture was electrophoresed on an 8% urea-denatured polyacrylamide gel at 200 V for 3 hr. The results were quantified by liquid scintillation counting [24]. The relative activity of DTD gene expression was normalized to the internal control of β-actin activity.

Nuclear extract preparation. The nuclear extracts were prepared according to the procedure of Dignam *et al.* [27], as modified by Benjamin and coworkers [28]. The cells were lysed in buffer containing 25 mM HEPES, pH 7.5, 70 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM $Na_3VO_4$, 0.4 mM NaF, 0.5 mM PMSF, and 1.0 mM DTT on ice for 20 min followed by extraction in 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 $Na_3VO_4$, 0.1 mM PMSF and 1.0 mM DTT with constant shaking at 4° for 4 hr. After centrifugation of the samples at 55,000 *g* for 1 hr at 4°, the supernatant was dialyzed in buffer containing 20 mM HEPES, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 10% glycerol, 0.4 mM NaF, 0.4 mM $Na_3VO_4$, 0.1 mM PMSF, and 1.0 mM DTT. The protein content of the samples was assayed by the Bradford assay (Bio-Rad, Richmond, VA).

NF-κB element oligonucleotide labeling. The NF-κB element oligonucleotides were synthesized by the Oligonucleotide Synthesis Facility at Fox Chase Cancer Center. A 33 base pair oligonucleotide located at 808-841 upstream (containing NF-κB consensus sequence) of the DTD 5′-flanking region (see Fig. 1), searched from the Gen Bank database of the DTD gene 5′-flanking region [8], was used.

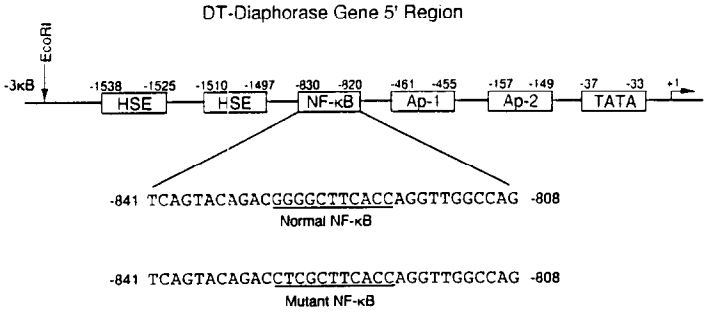


Fig. 1.Transcription factor binding elements in DTD gene 5′-flanking region. The position of the NF-κB binding site is shown in detail. The nucleotide sequences of the normal and mutant NF-κB binding site (denoted by underline) are shown.

A mutant NF-κB was constructed by altering the NF-κB sequence (see Fig. 1). Complementary DNA strands were purified and annealed according to standard procedure [29]. The double-strand oligonucleotides were end-labeled with [γ-$^{32}$P]ATP using $T_4$ polynucleotide kinase, followed by ethanol precipitation to remove the bulk of the unincorporated radioactivity.

Electrophoretic mobility shift assay. The end-labeled NF-κB oligonucleotide probe (20,000 cpm) corresponding to 0.25 ng of probe was mixed with 1.0 μg of poly(dI-dC) and 15 μg of nuclear extracts in a 30-μL final volume of binding buffer (20

mM HEPES, pH 7.5, 60 mM KCl, 1.0 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DDT, 0.01% Nonidet P-40, 0.1 mg/mL BSA, and 4% Ficoll). After incubation at room temperature for 25 min, the complexes were resolved by electrophoresis at 4° for 1 hr 45 min at 200 V. The gel was dried under vacuum and autoradiographed overnight at -80°.

Immunodepletion assay. Immunodepletion assay was carried out according to a standard procedure [30]. The nuclear extract was incubated with NF-κB p50 or p65 rabbit anti-serum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4° for 1 hr. The immune complexes were removed by immunoprecipitation and used for SDS-PAGE analysis; the supernatant was used in the electrophoretic mobility shift assay for stimulation of NF-κB DNA binding activity as described above.

## RESULTS AND DISCUSSION

Resistance to cytotoxic therapy has been associated with hypoxia [1,31,32]. In studies to explore the biological basis for this phenomenon, we found that the expression of several detoxication enzymes was increased as a direct response to hypoxic exposure [15]. The time-courses of the increase in enzyme activity and in mRNA expression were similar, and were delayed for some 24-48 hr after treatment. The time-course of induction of DTD is illustrated in Fig. 2. A similar time-course is observed in the induction of DTD by the dithiolthione oltipraz (Fig. 2A). This agent, a structural analogue of substances contained in cruciferous vegetables, is being developed as a chemoprotective drug. Davidson and colleagues [33] have shown that oltipraz administration results in the induction of transcription of GSH transferase genes. The administration of oltipraz to humans has been shown to induce transcription of DTD in peripheral mononuclear and colon mucosal cells in a clinical trial [34]. The mechanism of transcriptional induction by oltipraz has not yet been elucidated.

The time-course observed following a 4-hr treatment with MMC, which also resulted in DTD mRNA accumulation, resembled that following hypoxia and oltipraz. We have found that message accumulation following MMC treatment may also result from transcriptional induction, and that altered expression of the immediate-early genes *jun* and *fos*, and binding to the AP-1 element are associated phenomena (Hageboutros A, Yao K-S, Ford PA and O'Dwyer PJ, manuscript in preparation). The effect, which may be similar to jun-mediated response of the cell to DNA damage from UV radiation [35], was manifest within 4 hr of treatment and continued for 48 hr (Fig. 2A). The quantitative changes induced by each stimulus were similar at 24 hr, and represented an approximately 5-fold elevation in steady-state mRNA content (Fig. 2B).

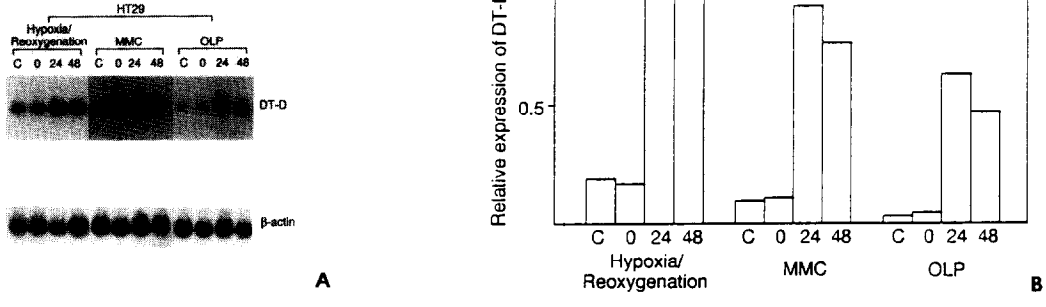


Fig. 2. Effects of hypoxia (8 hr), MMC (1.25 μM) and oltipraz (OLP, 100 μM) treatment on expression of DTD gene in human HT29 cells. The figure (A) shows 20 μg of cellular RNA from control (C), MMC- and OLP-treated cells hybridized to a human DTD cDNA probe that detects the 1.2 kb message shown. The control lanes (C) represent RNA from untreated cells. The β-actin probe was used as an internal control. The duration of treatment in hypoxia was 8 hr, followed by reoxygenation for 0, 24, or 48 hr; the MMC treatment was for 4 hr, and the OLP treatment was for 24 hr, followed by recovery in drug-free medium for the times indicated. The graph (B) shows the quantitative changes in expression as measured by laser densitometry, and normalized to the β-actin signal.

Whereas in HT29 cells a 5-fold elevation of DTD mRNA was observed during the period of reoxygenation following hypoxia [15], a more pronounced elevation occurred in the hepatoma-derived cell line HepG2 (Fig. 3). A 10-fold induction of DTD was observed, with a peak at 48 hr. The similarity of the time-course to that in HT29 cells was evident. We have described previously the involvement of AP-1 in the transcriptional response to both hypoxia/reoxygenation and MMC [16,36]. However, the induction of AP-1 binding (and of the transcription of *jun* and *fos* family members) is an early event during and after a hypoxic exposure, whereas the induction of DTD is delayed, with a peak at about 24 hr. Therefore, the involvement of additional promoter elements (possibly activated during the reoxygenation phase) was sought. The apparent redox responsiveness of NF-κB was potentially relevant to the action of each of these stimuli [18]. Others have identified the participation of both AP-1 and NF-κB in cellular responses to redox stress [37]; a synergistic interaction between NF-κB and other transcription factors has also been described [38].

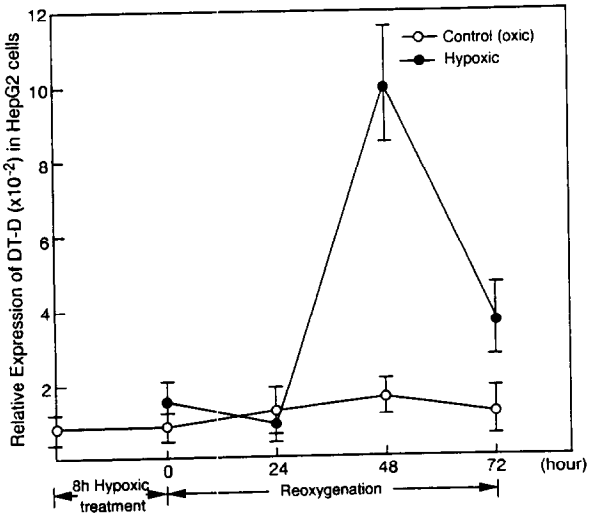


Fig. 3. Expression of DTD mRNA in HepG2 cells under oxic (o) or hypoxic (•) conditions. Cells were rendered hypoxic for 8 hr, and subsequently cultured under oxic conditions (reoxygenation) for the times indicated (hr). Quantitation was achieved by the reverse transcriptase-polymerase chain reaction method, and results are expressed relative to the expression of β-actin. Values are means ± SD, N = 4.

The 5′-flanking regions of the DTD gene in both rats and humans contain a single NF-κB-like element located -1035 bases and -820 bases upstream of the start site, respectively. The double-strand probe used in our studies was constructed using the human sequence derived from HepG2 cells (Fig. 1). The mutant probe altered the highly conserved guanine and cytosine residues at either end of the response element. Nuclear extracts from HT29 colon adenocarcinoma cells treated with hypoxia (followed by reoxygenation), oltipraz or MMC were found to contain proteins that retarded the NF-κB probe, with an intensity and time-course that were concordant with the northern analysis (Fig. 4). In both hypoxic and MMC-treated cells, the development of a slower mobility complex at 48 hr after the stimulus may reflect the participation of other rel family members in the NF-κB dimer. However, their late appearance does not suggest that their induction is a direct consequence of the inducing stimulus. Oltipraz-treated HT29 cells contained a complex with similar mobility, which was induced earlier (24 hr) and disappeared by 48 hr.

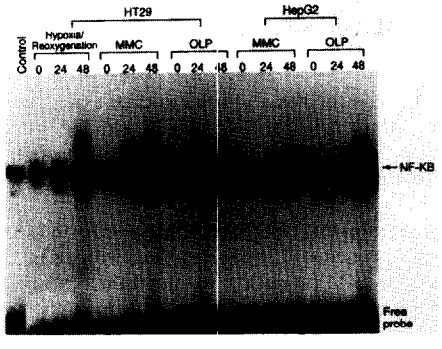


Fig. 4. Time-course of effects of hypoxia/reoxygenation, MMC and oltipraz (OLP) treatment on NF-κB element binding activity in nuclear extracts from cultured human HT29 and HepG2 cells. The times and experimental conditions are identical to those in Fig. 2. Electrophoretic mobility shift assays were carried out using a 33 bp double-strand oligomer containing the consensus DTD NF-κB element as the probe. The unbound probe is indicated at the bottom (free probe). The hypoxia, MMC and OLP-inducible DNA-protein complexes formed are indicated (arrow).

In contrast, in the HepG2 cells, there were two bands observed in control extracts. In MMC-treated cells, there was a loss of the lower band at 24 hr, and induction of both at 48 hr. The MMC response increased to a maximum at 48 hr, but there was no evidence of the induction of the slower mobility complex in HT29 cells. The peak oltipraz effect in HepG2 cells was also delayed to 48 hr, and the slower mobility complex was not seen clearly. This liver-derived cell line had higher constitutive DTD levels than HT29, and it exhibited a more florid elevation of steady-state DTD mRNA content following oltipraz treatment than that observed in HT29 cells (Fig. 3). It is possible that the late appearing, slower mobility complex is a negative regulator of transcription, since its appearance is followed by attenuation of the steady-state mRNA levels.

The specificity of the binding for NF-κB is shown by the competition experiment (Fig. 5). A mutant probe (Fig. 1) failed to show retardation by nuclear proteins. Competition for the DTD NF-κB element by unlabeled native NF-κB sequences eliminated protein binding to the labeled probe, showing that at a 1:10 ratio (pmol/pmol) the $^{32}$P-labeled NF-κB activity fell partially and at a 1:100 ratio, no binding activity was detected (Fig. 5) in MMC-treated nuclear extracts.

The p50 and p65 that constitute NF-κB bind DNA as dimers, either as p50 homodimers or p50-p65 heterodimers [20]. The nature of the proteins that comprise the nuclear extracts was investigated using antibodies specific for both p50 and p65 (Fig. 6). Preincubation of HT29 nuclear extracts with both antibodies resulted in depletion of the binding activity following each of the treatments. Thus, both proteins participated in the observed DNA-binding activity. The slower mobility band was not observed in this experiment, but its degree of retardation suggests the participation of additional peptides. Alternative higher molecular weight forms of p65 have been described [39]. A higher molecular weight complex would have the appearance of the slower mobility complexes identified above (Fig. 4).

These data indicate that the NF-κB response element may participate in the cellular response to various stimuli that result in the induction of detoxicating enzyme expression in both HT29 and HepG2 cells. The later induction of NF-κB binding is more closely related to the elevation of DTD activity than is the appearance of AP-1 binding, which is an early event occurring within 4 hr ([16], and unpublished data). The frequent finding of both elements in the promoter regions of detoxication and other protective genes suggests that there may be an interaction between them. We have demonstrated

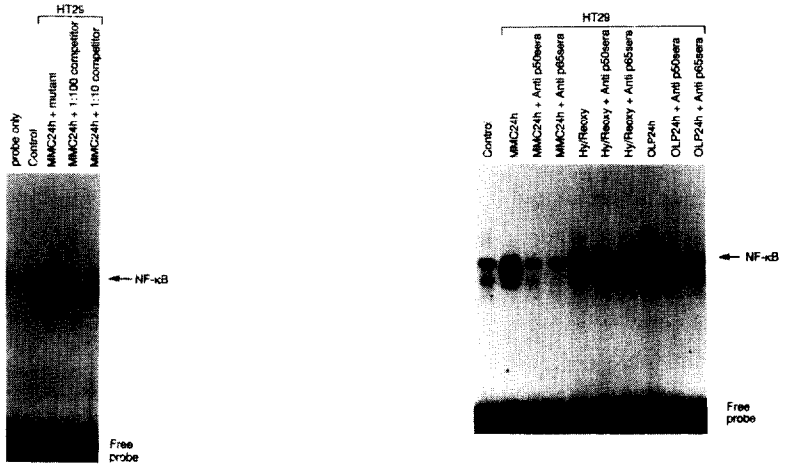


Fig. 5. Competition for NF-κB-binding activities in MMC-treated cells. The control lane represents nuclear extract from MMC-treated HT29 cells incubated with the NF-κB oligonucleotide probe. MMC-inducible DNA binding activity was not abolished by an identical concentration of a 33 bp oligomer containing a mutant DTD-NF-κB binding element (mutant), but eliminated completely with a 100-fold, and partially with a 10-fold molar excess of unlabeled natural NF-κB oligonucleotide (1:100, 1:10). (left figure)

Fig. 6. Immunodepletion assay of hypoxia, MMC and oltipraz (OLP) treatment on DTD NF-κB binding activity in HT29 nuclear extracts. The control lane contained 15 μg nuclear extract from unstimulated HT29 cells incubated with the 33 bp DTD NF-κB oligonucleotide. Preincubation of anti-p50 and anti-p65 sera with 15 μg nuclear extract from HT29 cells treated with hypoxia, MMC and OLP followed by precipitation of immunocomplexes was carried out before mobility shift assay analysis. The supernatant after removal of the immunocomplex was used for electrophoretic mobility shift assays and resulted in depletion of the NF-κB binding activity. (right figure)

that these stimuli induce the overexpression of other detoxicating enzymes, including in particular γ-glutamylcysteine synthetase in HT29 cells [6,15]. Studies to further characterize the possible interactions between these transcription factors are in progress.

In contrast to AP-1, transcriptional activation by NF-κB represents an effect exerted through cytoplasmic proteins. The major NF-κB subunits p50 and p65 exist in an inactive form in the cytoplasm bound to I-κB. As pointed out by Devary *et al.* [40], the cytoplasmic location of NF-κB implicates a plasma membrane- rather than a nucleus-initiated response. Koong *et al.* [41] have demonstrated recently the activation of NF-κB in Jurkat T cells exposed to only 3 hr of hypoxia. They demonstrated the degradation of cytoplasmic I-κBα as NF-κB binding activity increased, and associated the effect with tyrosine phosphorylation on the I-κB. Pretreatment of the cells with tyrosine kinase inhibitors, and transfection with a dominant negative mutant of raf-1 (a serine-threonine kinase) abolished I-κB degradation. Therefore, the hypoxic response in this instance appears to involve protein phosphorylation. The relevance of such a signal transduction pathway for the action of oltipraz and MMC is of interest, and its interruption may have therapeutic implications.

These investigators also point out that such a pathway may contribute to the response of tumors and other cells to hypoxia or to the cytokines that may be elaborated. In addition, the induction of protective enzymes, as demonstrated here, may enable the cells to survive hypoxic or other stress. It is of some interest that oltipraz and MMC appear to avail of the same pathway to generate a similar level of protection. The results therefore support the possibility that NF-κB may have a regulatory role in controlling the induction of protective genes in response to changes in the ambient environment. The elucidation of the pathways involved may provide novel avenues for both the treatment and the prevention of cancer.

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