NF-κB Activation by the Chemopreventive Dithiolethione Oltipraz Is Exerted through Stimulation of MEKK3 Signaling*

Chu Won Nho and Peter J. O’Dwyer‡

From the Division of Hematology-Oncology, School of Medicine and Abramson Cancer Center, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Received for publication, August 14, 2003, and in revised form, March 24, 2004
Published, JBC Papers in Press, March 26, 2004, DOI 10.1074/jbc.M309022200

Chemoprevention by the dithiolethione analogue oltipraz (4-methyl-5-(2-pyrazinyl)-1,2-dithiole-3-thione) may occur through several mechanisms, among them stimulation of detoxication activity. The phase II detoxification enzyme, NAD(P)H:quinone oxidoreductase 1 (NQO1; EC 1.6.99.2) also known as quinone reductase (QR) is well established to undergo transcriptional activation following oltipraz treatment of colon cancer cells in culture. Promoter analysis of the QR gene in oltipraz-treated cells reveals the involvement of both the AP-1 and NF-κB elements in the response. The emerging role of NF-κB in cell survival prompted a fuller analysis of effects of oltipraz on this pathway. Oltipraz treatment of both HCT116 and HT29 cells results in the induction of proteins involved in both pathways of NF-κB activation, including p65, IκB kinase α (IKKα), IκB kinase β (IKKβ), and NF-κB-inducing kinase (NIK). IκBα total protein levels were unchanged, but phosphorylation of the inhibitor was also induced in both lines. Electrophoretic mobility shift assay (EMSA) analysis confirmed induction of protein binding to a consensus NF-κB element, and transcriptional activation was further confirmed using a reporter construct. Transcriptional activation of NF-κB was decreased in a dose-dependent manner by dominant-negative NF-κB in both cell lines. The molecular mechanism that triggers IKK activation in response to oltipraz was also examined using inhibitory constructs of NIK and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 3 (MEKK3). We found that both MEKK3 and NIK exert effects on IKKα/β activation, but through different pathways. Furthermore, the receptor-interacting protein (RIP) was found to interact strongly with MEKK3 during oltipraz-induced NF-κB signaling, implying a role for tumor necrosis factor receptor signaling in the action of oltipraz. These results implicate a novel signaling pathway for the action of oltipraz in QR gene regulation.

Prevention of cancer through the administration of inexpensive non-toxic molecules has the potential to decrease the incidence of this important disease (1). Compounds with established success include anti-estrogens for breast cancer prevention (2, 3), aspirin for colon cancer prevention (4), and retinoids to prevent second primaries on patients with head and neck cancer (2). Micronutrients and other natural products are being explored in other cancers. Novel candidate molecules with defined chemopreventive mechanisms are needed.

Oltipraz is a synthetic dithiolethione with experimental support for its role as a chemoprevention agent. Dithiolethiones are constituents of the Brassica family of green leafy vegetables, high consumption of which has been shown to lower cancer risk (5, 6). Cruciferous vegetables of the Brassica family, such as broccoli and Brussels sprouts, appear to be most effective in this regard (7, 8). Oltipraz has several proposed mechanisms of action, including induction of detoxication pathways (9), DNA repair stimulation (10), and alteration of the expression of additional genes that may relate to carcinogenesis (11). Studies in various models of carcinogenesis have shown protection by oltipraz from the induction of cancers in skin, breast, bladder, lung, colon, pancreas, stomach, and liver (11). Clinical trials have established that induction of detoxication pathways can be achieved at tolerable doses of oltipraz (12), though a large scale study in China produced mixed results (13). Furthermore, doses that produce pharmacological activity are also associated with some skin and neurological toxicity that limit the therapeutic potential of the agent (14). It is critical therefore to understand the mechanism of chemoprevention by oltipraz so that targets for this activity can be identified, and candidate molecules with an improved toxicity profile tested against them.

We have previously examined the effects of oltipraz on regulation of detoxication enzymes, including glutathione transferases and quinone reductase (15). We also have reported that transcriptional regulation of the QR gene involves the stimulation by oltipraz of a number of transcription factors, including activating protein-1 (AP-1), and nuclear factor kappa-B (NF-κB) (16, 17). Although NF-κB alone seems to account for only 20–30% of QR promoter activity revealed by mutational analysis, NF-κB is responsible for a proportion of the immediate response on the QR gene promoter, and it is of interest based on the pleiotropic range of cells and promoters in which it is active. In addition, the anti-apoptotic role of NF-κB in many transformed cells may have implications for its potential role in chemoprevention.

The NF-κB family of transcription factors were identified as

* This work was supported by Grant CA-78272 from the NCI, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: School of Medicine, 51 N, 39th St., MAB-103, Philadelphia, PA 19104. Tel.: 215-682-8636; Fax: 215-243-3269; E-mail: peter.odwyer@uphs.upenn.edu.

1 C. W. Nho, manuscript in preparation.

2 The abbreviations used are: QR, quinone reductase; NF-κB, nuclear factor-κB; IKK, IκB kinase; MEKK3, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 3; RIP, receptor-interacting protein; TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor; nt, nucleotide; ELISA, enzyme-linked immunosorbent assay; TRADD, TNF receptor-associated death domain; EMSA, electrophoretic mobility shift assay; HA, hemagglutinin.
regulators of genes involved in coordinating immune responses, and subsequently found to be active in other cell types. NF-κB is composed of homo- and heterodimers of five members of the Rel family including RelA (p65), RelB, c-Rel, NF-κB1 (p50), and NF-κB2 (p52). Two mechanisms for their activation have been described. In one, NF-κB proteins are present in the cytoplasm in an inactive form, bound by inhibitor molecules that include IκBα, IκBβ, and IκBe. In the second, the inactive precursor forms of p50 and p52, p105 and p100 respectively are present unassociated with the inhibitors (18). Activation of NF-κB may be triggered by a signaling cascade that results in phosphorylation of IκB by an IκB kinase (IKK) complex composed of IKKα, IKKβ, and IKKγ, the activity of which results in the release and subsequent proteasomal degradation of IκB (19). In the alternative pathway, partial degradation of the p100 precursor by proteolysis releases active p52, which permits translocation to the nucleus of preformed p50/REB complexes (20, 21). The enzyme responsible for the activation of p100 proteolysis is NIK (22), which activates IκKα, which in turn acts on p100 to direct it toward proteasomal processing and release of p52 (23, 24). This alternative pathway has shown to be involved in signaling cascades that act in the development, organization, and function of lymphoid tissue.

The molecular mechanism(s) by which the IKK complex is activated is an active area of current investigation. There are several potential upstream IKK kinases, including MEKK1 (25), MEKK2 (26), MEKK3 (26), NIK (27), TNF receptor-associated factor (TRAF) binding kinase 1 (TBK1), also known as TRAF2-associated kinase; T2K, or NF-κB-activating kinase, NAKO (28) and transforming growth factor-β-activating kinase (TAK1) (29), all of which have been shown to phosphorylate and activate IKK directly in vitro. A recent study confirmed that MEKK3 is essential for TNF-induced NF-κB activation (30). The authors found that MEKK3 acted downstream of TRAF2 and receptor-interacting protein (RIP). In addition, MEKK3 was found to interact with RIP physically (37).

To pursue further the role of NF-κB in QR gene regulation and more broadly in chemoprevention, we investigated the effects of oltipraz treatment on the upstream events that signal NF-κB transactivation. We report here that NF-κB activation by oltipraz in colon cancer cells occurs primarily by activation of MEKK3 followed by IKKα and IKKβ activation. Evidence of activation of NIK was also demonstrated, but signaling through p100 did not appear to be important. A possible role for TNF-α receptor signaling in mediating the pharmacological effect is suggested by the involvement of the RIP-MEKK3 complex in the response.

**EXPERIMENTAL PROCEDURES**

**Reagents and Constructs—**Oltipraz was purchased from LKT Labs (>99% purity, St. Paul, MN) and dissolved in MeSO (stock solution is 50 mM). NF-κB was purchased from BD Biosciences (San Diego, CA) and dissolved in MeSO (stock solution is 100 μg/ml). The NF-κB reporter plasmid containing four copies of an NF-κB-binding site (pNF-κB-Luc) and its control vector (pTAL-Luc) were purchased from Clontech (Palo Alto, CA). The structure of the human pBD890-CAT construct has been described previously (31). pCATT-Basic vector, pCMV-β-gal vector, and pRL-CMV were purchased from Promega (Madison, WI). Mammalian expression vectors for HA-tagged RIP-1 (p300) and RIP-3 (p68–81) were provided by Dr. Tatsushi Miyashita (National Research Institute, Tokyo, Japan). Dominant-negative mutants of NF-κB signaling, IκB, and dnNIK (IKKδ24missAA240) were provided by Dr. Karen Vousden (NCI, National Institutes of Health, Bethesda, MD).

**Cell Lines and Cell Treatments—**HT29 and HCT116 colon adenocarcinoma cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% air/5% CO2. Cells were plated at a density of 5 × 104 cells per 100-mm dish 24 h prior to 100 μM oltipraz or 0.02% MeSO treatment (solvent control). The cells were exposed to oltipraz for 24 h, after which oltipraz was removed, and fresh medium added for further incubation. Total RNA or protein extracts were isolated from the cells at various times (0, 6, 24, 30, and 48 h).

**Cytosolic and Nuclear Extract Preparation—**Nuclear and cytosolic proteins were prepared using CelLytic™ NuCLEAR™ Extraction Kit (Sigma) according to the manufacturer’s manual. Briefly, the cells were washed with cold phosphate-buffered saline and collected in conical centrifuge tubes. After a 5-min centrifugation, the pellet was resuspended in 150 mM hypotonic lysis buffer containing the protein inhibitors: aprotinin (2 μg/ml), leupeptin (2 μg/ml), pepstatin (5 μg/ml), 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol. The resuspended cells were centrifuged at 420 × g for 5 min 4 °C. The pellet was again resuspended with the lysis buffer and disrupted using a glass tissue homogenizer. The disrupted cells were centrifuged for 30 min at 20,000 g at 4 °C, and the supernatant saved as a cytosolic extract. The pellet was resuspended in extraction buffer (20 mM Heps, pH 7.9, 1.5 mM MgCl2, 0.42 mM NaCl, 0.2 mM EDTA, 25% glycerol) containing the protein inhibitors: aprotinin (2 μg/ml), leupeptin (2 μg/ml), pepstatin (5 μg/ml), 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol. The resuspension was incubated on ice for 30 min with gentle shaking and centrifuged at 20,000 g for 5 min. The supernatant was stored as a nuclear extract at −80 °C.

**Western Blotting—**Cytosolic or nuclear protein (10–15 μg per well) was separated on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes for Western blot analysis. Antibodies against p65 (RelA), p50, IκBα, NIK, and NQO1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). IKKα, IKKβ, and p-1-B-Body was purchased from Cell Signaling Technology (Beverly, MA); and anti-MEKK3 antibody was purchased from BD Transduction Laboratories (San Diego, CA). A blocking monoclonal antibody against TNF-R1 (clone 55R-170) was purchased from R & D Systems (Minneapolis, MN). HCT116 cells were incubated with TNF-R1 blocking antibody (25 μg/ml) for 1 h prior to TNF-α (100 ng/ml) or oltipraz (100 μM) treatment for 1 or 24 h, respectively. Following treatments, the cells were harvested and subjected to Western blotting with specific antibodies against TRADD, RIP, TRAF2, and IκBα. TRADD and TRAF2 antibodies were purchased from BD Transduction Laboratories.

**Northern Blotting—**Total RNA was isolated using TRIzol reagent (Invitrogen) following the manufacturer’s instructions. Total RNA (15 μg) was electrophoresed, transferred onto nitrocellulose membranes, and hybridized to a 1.2-kb human quinone reductase probe (32) at 65 °C. Membranes were washed for 1 h in 2 × SSC (1 × SSC: 0.13 M NaCl, 0.03 M sodium citrate, pH 7.0) containing 0.5% SDS and exposed to autoradiography.

**Electrophoretic Mobility Shift Assay (EMSA)—**The NF-κB site in the human quinone reductase promoter region, 5′-TCAGTCACAGC-3′ was first labeled with [γ-32P]ATP using polynucleotide kinase (Promega). 10 μg of nuclear extracts were incubated with gel shift binding buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 0.25 μg/ml poly(dI:dC), 20% glycerol) for 10 min at 20 °C, and then 1 μg of control Oct-1 probe was labeled with [γ-32P]ATP (greater than 50,000 cpm/ml), and samples were incubated for a further 20 min at room temperature. The samples were separated on a 6% polyacrylamide gel in 0.5× Tris borate/EDTA buffer at 200 V. The gels were dried and developed by autoradiography.

**Transfection and Reporter Gene Analysis—**HT29 and HCT116 cells were plated at 5 × 104 cells per well in 8-well plates 24 h prior to transfection. The cells were transfected using the FuGENE transfection reagent (Invitrogen) with 1 μg of pNF-κB-Luc or 1 μg of pTAL-Luc, in combination with 0.1 μg of pRL-CMV vector (Promega). pRL-CMV directly expresses Renilla luciferase under a constitutive promoter and was used as an internal control to normalize the obtained NF-κB responses. After 24 h of transfection, cells were washed with fresh medium and then exposed to 500 μM oltipraz or 1 μg/ml of pTAL-Luc or pNF-κB-Luc vector for 24 h. After 24 h, the cells were harvested, and luciferase activity was measured in a luminometer (Turner Design, Sunnyvale, CA). The relative light units (RLUs) were calculated as the ratio of firefly luciferase to Renilla luciferase luminescence. Nonspecific luciferase activation was determined in parallel experiments with pTAL-Luc (control vector) and deducted. Normalized luciferase activities are presented as fold induction relative to the activity obtained from the MeSO control group. The cells were transfected as above with 1 μg of pBD890-CAT or 1 μg of its control vector (pCAT-Basic vector) in a combination with 0.5 μg of pCMV-β-gal. CAT protein production was determined using the CAT-ELISA kit (Roche Applied Science) according to the manufacturer’s instructions. The cells were harvested, and CAT activity was measured in ELX 800 Universal Microplate Reader (BIO-TEK, INC). Nonspecific CAT activation was determined in parallel experiments with pCAT-Basic (control vector) and was deducted. The transfection efficiency between samples was corrected with values of β-galactosidase expres-
Oltipraz Activates NF-κB in QR Regulation

Oltipraz Activates NF-κB in QR Regulation

**RESULTS**

Oltipraz Induces NF-κB Signaling Pathways—Although most of the individual regulatory elements (including AP-1 and NF-κB binding sites) have been identified in the QR promoter region (31, 33), the particular signaling cascades involved in its regulation have yet to be described. A few reports have defined the upstream signals for detoxication enzyme activation upon treatment with dietary components such as isothiocyanates (34, 35). In our previous work we found that NF-κB is one of the key transcription factors involved in quinone reductase gene regulation by oltipraz (16, 31), though the upstream signaling events leading to NF-κB activation have not been described. To analyze the basis for NF-κB activation, we investigated the expression of key proteins involved in its activation pathway. Increases in the cellular content of p65, p50, IKKα, and IKKβ occurred in a time-dependent manner following exposure to 100 μM oltipraz in both HT29 and HCT116 cells (Fig. 1). IkBα degradation was not observed, but phosphorylation of IkBα was markedly increased in a time-dependent manner. This finding is consistent with previous reports suggesting that incomplete degradation of IkBα is sufficient for NF-κB activation in certain types of colon epithelial cells including HT29 cells (36, 37). To analyze the functional status of NF-κB protein complexes induced by oltipraz, we performed EMSA assays. The binding activity on the NF-κB binding site in the regulatory region of QR was increased by oltipraz in both cell lines (Fig. 2, top panel). The binding activities gradually increased from 6 to 48 h post-treatment. No effect of oltipraz was observed on binding to the Oct-1 element (Fig. 2, lower panel). To confirm transcriptional activation of the NF-κB element in QR by oltipraz, we conducted a transfection experiment using a reporter construct containing the NF-κB element found in the regulatory region of the human QR gene (Fig. 3A). NF-κB activation was significantly induced in both HT29 and HCT116 cells, with minor differences between them in the degree of the response. To provide further functional evidence for NF-κB involvement in oltipraz-induced signaling, we used a dominant-negative mutant of NF-κB (IkB, IkB superrepressor) and a dominant-negative mutant of the upstream kinase, dnNIK (dominant-negative NIK). IkB cannot be phosphorylated and therefore precludes degradation of IkB. dnNIK, is a kinase-inactive mutant of NIK that lacks the two lysine residues in its catalytic domain (NIK(K429->430AA)), and so behaves as a dominant-negative inhibitor to suppress NF-κB activity. HCT116 cells were co-transfected with the pNF-κB-Luc vector together with various concentrations of IkBSR or dnNIK or its empty expression vector, pcDNA3.1. After 24 h of transfection, the cells were treated with 100 μM oltipraz for an additional 24 h and analyzed for luciferase activity. Oltipraz-induced NF-κB transcriptional activity was decreased by IkBSR and dnNIK in a dose-dependent manner, and 1 μg of IkBSR or dnNIK completely abrogated the effect of oltipraz on NF-κB activation in HCT116 cells (Fig. 3B). Taken together, these results confirm that oltipraz clearly induces NF-κB signaling in colon cancer cells, and
Oltipraz Activates NF-κB in QR Regulation

Fig. 2. Oltipraz increases NF-κB binding activities. Nuclear protein was extracted from the cells (5 × 10^6) treated with MeSO (0 h control) or 100 μM oltipraz for 6, 24, 48 h in HT29 and HCT116 cells. 10 μg of nuclear extract from each sample was used for EMSA using either NF-κB (top panel) or Oct-1 (lower panel) oligonucleotide as probes. Specific anti-p65 antibody was added to the reaction mixtures of 24 h-treated cells prior to incubation with the labeled probe (lanes 6 and 12). A competition experiment was also performed with a 100-nolar excess of unlabeled NF-κB oligonucleotide (lanes 5 and 11). Results from one of five independent experiments are shown.

Fig. 3. Oltipraz increases the transcriptional activation of NF-κB. A. Effect of oltipraz on NF-κB transcriptional activation in HT29 and HCT116 cells. The cells (5 × 10^6) were co-transfected with 1 μg of pNF-κB-Luc or 1 μg of pTAL-Luc vector, in a combination with 0.1 μg of pRL-CMV vector for 24 h and then treated with MeSO (control) or 100 μM oltipraz for an additional 24 h. After the treatment, cells were lysed and analyzed using a Dual-Luciferase Reporter System as described under “Experimental Procedures.” Normalized luciferase activities were presented as fold induction relative to the activity obtained after treatment with pTAL-Luc, a construct that contains the regulatory region (from −1 to −890) of the human QR gene. As shown in Fig. 4A, co-transfection of pDTD890-CAT with the IκBα or dnNIK construct noticeably decreased the oltipraz-induced CAT activity although it was not statistically significant (p = 0.06). This result is consistent with our recent report suggesting that NF-κB alone accounts for only 20–30% of QR promoter activity in oltipraz-treated colon cancer cells, which was revealed by mutational analysis of the QR promoter region (17). We also determined the level of QR mRNA in the colon cancer cells after co-transfection of the inhibitory constructs, IκBα and dnNIK. We found a dose-dependent decrease in mRNA levels by both dominant-negative mutants in oltipraz-treated HCT116 cells (Fig. 4B). The cells transfected with 3–9 μg of the constructs even showed a statistically significant decrease in QR mRNA expression compared with the cells transfected with empty expression vectors (Fig. 4C). This result indicates the importance of upstream signaling to NF-κB to determine QR expression after oltipraz treatment.

NIK and MEKK3 Are Both Involved in NF-κB Regulation by Oltipraz—Several MAP3K-related kinases including NIK and MEKK3 have been suggested to play a role in TNF-induced NF-κB regulation (30, 38). To examine whether any of these kinases are involved in NF-κB activation by oltipraz, we measured NIK and MEKK3 protein expression changes following oltipraz treatment in HCT116 cells. The expression of both NIK and MEKK3 was increased by oltipraz in a time-dependent manner (Fig. 5A). In order to confirm that both NIK and MEKK3 are essential for NF-κB activation, we transfected HCT116 cells with dnNIK or MEKK3-siRNA or its own expression vector, together with pNF-κB-Luc (Fig. 5B). MEKK3-siRNA was generated from a single nucleotide mismatch in a 19-nt sequence targeting MEKK3, and it suppressed MEKK3 expression compared with control transfected with the empty expression vector, pSilencer 1.0-U6 (Fig. 5B). Both of these inhibitory approaches caused a significant decrease in oltipraz-induced NF-κB transactivation (Fig. 5C). These results confirm that both NIK and MEKK3 are crucial signaling intermediates in oltipraz-induced NF-κB activation.

Both IKKα and IKKβ Are Activated by MEKK3 and NIK—There is good evidence that MEKK3 and NIK can activate IKKα and IKKβ (26, 30), but the specific substrate specificity of each kinase is a matter of some controversy, and it is clear that the degree of activation of either IKKα or IKKβ may be dependent upon the nature of the external stimulus (39). To determine which IKK is affected by NIK and MEKK3 activation, we measured IKKα and IKKβ protein expression and kinase activity after transient transfection of dnNIK and MEKK3-siRNA in oltipraz-treated HCT116 cells. The cells were co-transfected with pSilencer 1.0-U6 (Fig. 6A). Both dnNIK and MEKK3-siRNA treatment decreased the content of both IKKs, and the effect was greatest when the inhib-
Oltipraz Activates NF-κB in QR Regulation

Fig. 4. NF-κB is involved in QR gene regulation induced by oltipraz. A, inhibition of NF-κB signaling and QR promoter function. HCT116 cells (5 × 10⁵) were co-transfected with 0.5 μg of pCMV-β-gal vector and 1 μg of pDTD890-CAT or 1 μg of pCAT-basic control vector together with 1 μg of the IκBα or dnNIK, or the empty expression vector, pcDNA3.1. After 24 h of transfection, the cells were treated with MeSO₄ (control) or 100 μM oltipraz for an additional 24 h. The cells were then analyzed using a CAT-ELISA kit as described under “Experimental Procedures.” Normalized CAT activities were presented as fold induction relative to the activity obtained from the control group. The data shown are means ± S.D. of three independent experiments done in triplicate. B, effects of IκBαSR and dnNIK on QR protein levels in HCT116 cells. The cells (5 × 10⁵) were transfected with 3 μg of the empty expression vector, pcDNA3.1 or dominant negatives, IκBαSR or dnNIK as indicated. After 24 h of transfection, the cells were treated with MeSO₄ (control) or 100 μM oltipraz for an additional 24 h, and were analyzed by Western blotting using anti-NQO1 antibody. The numbers below each band represent the mean fold induction of protein levels based on densitometric analysis from three independent experiments. The statistical significance of the difference between the empty vector-transfected group and dominant-negative-transfected groups were assessed by the two-sample Student’s t test between two paired samples. One asterisk denotes the statistically significant difference with p values less than 0.05. C, effects of IκBαSR and dnNIK on QR mRNA levels in HCT116 cells. The cells (5 × 10⁵) were transfected with 3 μg of the empty expression vector, pcDNA3.1 or dominant negatives, IκBαSR or dnNIK as indicated. After 24 h of transfection, the cells were treated with MeSO₄ (control) or 100 μM oltipraz for an additional 24 h. Total mRNA was then extracted and Northern blot analysis performed using a human QR cDNA probe to detect two QR mRNA transcripts (upper and lower bands). Equal loading of mRNA was monitored by hybridizing the same membrane with a glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA probe. Results from one of three independent experiments are shown. D, quantitation of QR mRNA levels. Bar graphs (means ± S.D.) represent quantitation of QR mRNA levels shown above. Densitometry readings of QR mRNA (lower transcript bands) were normalized to G3PDH levels and expressed as % control mRNA. The statistical significance of the difference between the vector-transfected group and dominant-negative-transfected groups were assessed by the two-sample Student’s t test between two paired samples. One asterisk denotes the statistically significant difference with p values less than 0.05.

RIP Is Required for Oltipraz-induced NF-κB Signaling—RIP has been described to be a key effector for TNF-α-induced NF-κB activation (40). RIP is also known to be essential for the recruitment of IKK kinases in TNF-α-induced NF-κB signaling, although the kinase activity of RIP is not essential to this activation (41). Therefore, it is important to determine whether RIP is involved in the activation of NF-κB by oltipraz. We examined RIP expression by oltipraz and found a marked increase at 24 and 48 h post-treatment in HCT116 cells (Fig. 7A). We then tested whether RIP is required in oltipraz-induced NF-κB activation by using two different types of RIP deletion mutants: RIP-(1–300) and RIP-(569–671). The RIP protein contains three domains, including an N-terminal kinase domain, an intermediate domain and C-terminal death domain (42). RIP-(1–300) contains only the kinase domain and RIP-(569–671) contains only the death domain. After transfection, the cells were treated with 100 μM oltipraz for an additional 24 h and harvested for luciferase assay or Western blotting. The RIP deletion mutants significantly inhibited oltipraz-induced NF-κB transcriptional activation (Fig. 7B) as well as IκKα/β expression through TNF receptor signaling (Fig. 7C). Depletion of TRADD, and lack of effect on TRAF2, imply a selective effect of oltipraz on intracellular components of TNF receptor signaling. These data suggest that RIP is an essential factor in oltipraz-induced NF-κB activation.

MEKK3 Interacts with RIP in Oltipraz-induced NF-κB Signaling—It has been reported that MEKK3 and NIK are critical protein kinases for the TNF-α-induced activation of NF-κB (30, 41, 43). In addition, interaction between MEKK3 and RIP is known to be an important feature of TNF-induced NF-κB activation (30). Since we found here that both MEKK3 and NIK play a role in oltipraz-induced NF-κB activation, we further examined whether MEKK3 or NIK interacts physically with RIP in oltipraz-induced NF-κB signaling by immunoprecipitation experiments. Total cell extracts were immunoprecipitated with anti-RIP antibody, and the immunoprecipitates were analyzed by Western blotting using anti-MEKK3 or anti-NIK antibodies. As shown in Fig. 7D, MEKK3 was clearly present in the immunoprecipitates from the oltipraz-treated cells, whereas we observed a very weak interaction with NIK. These data show that MEKK3, but not NIK, signaling is exerted...
through an interaction with RIP, implicating TNF receptor signaling in the oltipraz response.

**Oltipraz Activates NF-κB through TNF-R1 Signaling**—The proinflammatory cytokine TNF exerts its pleiotropic function by binding to one of two receptors, TNF-R1 and TNF-R2 (44). Ligand binding induces receptor trimerization and recruitment of several signaling proteins to the cytoplasmic domains of the receptors. In the case of TNF-R1, the first recruited protein is TRADD, binding of which recruits additional adaptor proteins, including RIP, TRAF2, and FAS-associated death domain (FADD). Since oltipraz treatment triggered formation of the RIP–MEKK3 complex, we examined the role of TNF-R1 in oltipraz-induced NF-κB signaling using specific blocking antibodies. HCT116 cells were preincubated with TNF-R1 blocking antibody (25 μg/ml) (or none) for 1 h and were treated with either TNF-α (100 ng/ml) for an additional 1 h, or oltipraz (100 μM) for 24 h. The cells were then analyzed by Western blotting using specific antibodies against TRADD, RIP, TRAF2, and IKKβ (Fig. 8). We found that TNF-α produced small increases in TRADD, RIP, TRAF-2, and IKKβ that were abrogated by preincubation with TNF-R1 blocking antibody. Oltipraz treatment resulted in greater expression of all of these proteins, and the increases were attenuated but not completely abrogated by preincubation with the TNF-R1 antibody. Consistent with the data in Fig. 7, the major effects are on RIP and IKKβ, both of which increase with oltipraz treatment, an effect largely abrogated by receptor blockade. These data suggest that oltipraz-induced NF-κB signaling is exerted through activation of the TNF-R1 complex.

**DISCUSSION**

Oltipraz is a candidate chemoprevention agent that was developed based upon its ability to upregulate detoxication genes and so to protect normal cells from mutagenic insults (14). It was demonstrated early that the effect on detoxication genes is exerted through transcriptional induction, and various promoter elements have been shown to be important in this action (9, 31). Though oltipraz has a number of side effects that may impinge upon its value as a chemopreventive agent for broad use in the population, the importance of its mechanism of induction of detoxication genes as a means of protecting tissues is not in question. It is therefore critical to understand the biological pathways by which oltipraz exerts these effects. Our
previous studies showed that two cis-acting elements, AP-1 and NF-κB, are involved in human QR gene regulation (16, 31). In this report, we have focused on NF-κB regulation by oltipraz. NF-κB has been shown to play an important role in regulating anti-apoptotic and pro-apoptotic events, depending on the physiological circumstances (45). We show here that the NF-κB dominant-negative mutants clearly inhibited QR promoter activity and mRNA expression confirming that NF-κB is one of
the essential factors accounting for QR gene regulation in oltipraz-treated colon cancer cells.

To establish the upstream signaling mechanisms of NF-κB activation by oltipraz, we have examined the sequence of events that lead to QR expression in the colon cell model. We found that MEKK3 and RIP both play an essential role in oltipraz-induced NF-κB signaling. Our data suggest that MEKK3 was required for IKKα and IKKβ activation and that it interacted physically with RIP in mediating the activation. Several members of the mitogen-activated protein kinase kinase kinase (MAP3K) family including MEKK1 (25), MEKK2 (26), MEKK3 (26), NIK (27), TRB1 (28) and TAK1 (29) have been suggested to activate IKK when overexpressed. However, some of these do not appear to be physiologically relevant, since genetic studies reveal that absence of MEKK1 (46), and NIK in our study to test IKK activation in vivo. A recent study has reported that MEKK3 has a critical role for activation of TNF-induced NF-κB signaling (30). While NIK had been eliminated as a candidate IKK kinase in cellular models (40), we included NIK in our study to test IKK activation because the dominant negative of NIK had decreased QR mRNA levels (Fig. 4, B and C). Both MEKK3 and NIK showed similar potency to activate oltipraz-induced NF-κB transactivation, and while MEKK3 showed a somewhat greater ability to activate both IKKα and IKKβ than did NIK, both were active in this regard too (Fig. 6). Therefore, we believe that MEKK3 and NIK are essential kinases for IKK activation in oltipraz-induced NF-κB signaling.

Because RIP is known to be one of essential effectors in TNF-α-mediated IKK activation (41), we examined whether RIP is involved in oltipraz-induced NF-κB signaling by interacting with MEKK3 or NIK. Not only did we observe an essential role of RIP in oltipraz-induced NF-κB signaling confirmed by transfection experiments using RIP deletion mutants (Fig. 7, B and C), we also found a physical interaction with MEKK3 as measured by coimmunoprecipitation experiments with RIP (Fig. 7D). However, when we examined the physical interaction between RIP and NIK after oltipraz treatment, it was unchanged compared with the basal level (Fig. 7D). Although NIK has no significant interaction with RIP, it did show some effects on NF-κB and IKK activation in oltipraz-treated cells (Figs. 5C and 6, respectively). This result suggests that NIK may activate the IKKα/β complex independently of RIP, since NIK is known to interact with the IKKα/β heterocomplex form (27), and to activate IKKα/β as shown by NF-κB activation following overexpression of NIK (26, 48). Therefore in oltipraz signaling, two pathways to IKK activation are induced, one through the RIP-MEKK3 complex and the other through NIK by as yet undetermined signals.

We hypothesize based on these data that oltipraz-induced NF-κB signaling occurs through the TNF-α receptor 1 (TNF-R1) complex. This possibility is supported by the fact that the presence of the RIP-MEKK3 complex is required in the TNF-R1 complex for the activation of IKK in TNF-α-mediated NF-κB signaling (30). Although we observed a requirement for RIP and the presence of RIP-MEKK3 complexes in oltipraz-induced NF-κB signaling, further clarification of TNF-R1-dependent signaling by oltipraz was required. Thus, we investigated whether the TRADD and TRAF2 in addition to RIP are also involved in this pathway since both molecules are essential for TNF-R1-dependent NF-κB activation. We found that IKKβ and RIP levels were decreased after blocking TNF-R (Fig. 8). We also examined if oltipraz caused the cells to produce TNF-α for triggering TNF-R signaling. We found that there is no significant increase in TNF-α production by 100 μM oltipraz at any of time points tested (data not shown). This implies that oltipraz-induced TNF-R signaling is not a consequence of the autocrine action of TNF-α, but could depend on intracellular regulation of TNF-R signaling. Taken together, these data suggest that oltipraz triggers NF-κB signaling through intracellular TNF-R1 activation, the mechanism of which is currently under investigation. Since RIP and TRAF2 either directly or indirectly interact with both TNF-R1 and TNF-R2 for NF-κB transcriptional activation (40), TNF-R2 signaling is another plausible target for future investigation.

The activation of IKKα by NIK stimulated us to investigate the alternative pathway of NF-κB signaling via RelB/p100 processing described previously (49). In this non-classical pathway, the p100 precursor is bound by RelB in the cytoplasm and
Upon proteasomal processing (stimulated by IKKα), releases p52 in a RelB heterodimer that undergoes nuclear translocation and interaction with NF-κB-responsive genes. We found that there was no increase of RelB and p52 in nuclear extracts after oltipraz stimulation (data not shown), and so ruled out this pathway as an important component of the action of oltipraz in colon cells.

We also noted some differences among the two cell lines used as models in these experiments. NF-κB signaling involves various intermediate proteins and is a multistep process. We investigated expression of several of these proteins and found a significant increase in their levels by 100 μM oltipraz treatment in both HT29 and HCT116 cells (Fig. 1). It is interesting to note that all of the proteins examined showed higher expression in HCT116 cells compared with HT29 cells. We speculate that this may be due to the different p53 status between two cell lines: HT29 cells contain this is may be due to the different p53 status between two cell lines. We speculate that this pathway as an important component of the action of oltipraz after oltipraz stimulation (data not shown), and so ruled out this pathway as an important component of the action of oltipraz.

We also noted some differences among the two cell lines used as models in these experiments. NF-κB signaling involves various intermediate proteins and is a multistep process. We investigated expression of several of these proteins and found a significant increase in their levels by 100 μM oltipraz treatment in both HT29 and HCT116 cells (Fig. 1). It is interesting to note that all of the proteins examined showed higher expression in HCT116 cells compared with HT29 cells. We speculate that this may be due to the different p53 status between two cell lines: HT29 cells contain this is may be due to the different p53 status between two cell lines.

References

1. Sporn, M. B., and Suh, N. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9319–9324
2. Zhao, Q., and Lee, F. S. (1999) J. Biol. Chem. 274, 8355–8358
3. Wotton, J. D., Gao, X., Cao, Z., Rothe, M., and Goeddel, D. V. (1997) Science 278, 866–869
4. Bonnard, M., Mirtosos, C., Suzuki, S., Graham, K., Huang, J., Ng, M., Itie, A., Wakeham, A., Shahinian, A., Hencz, W. J., Eksi, A. A., Shillinglaw, W., Mak, T. W., Cao, Z., and Yeh, W. C. (2000) EMBO J. 19, 4976–4985
5. Nisnolmi-Tauji, J., Kishimoto, K., Hiyama, A., inoue, J., Cao, Z., and Matsumoto, K. (1999) Nature 398, 252–256
6. Yang, J., Lin, Y., Gao, Z., Cheng, J., Huang, J., Deng, L., Liao, W., Chen, Z., Liu, Z., and Su, B. (2001) Nat. Immunol. 2, 620–624
7. Yao, K. S., Hageboutros, A., Ford, P., and O'Dwyer, P. J. (1997) Mol. Pharmacol. 51, 422–430
8. Yao, K. S., Godwin, A. K., Johnson, C., and O'Dwyer, P. J. (1996) Cancer Res. 56, 1731–1736
9. Li, Y., and Fiswal, A. K. (1993) J. Biol. Chem. 268, 21454
10. Wilhelm, D., Bender, K., Knebel, A., and Angel, P. (1997) Mol. Cell. Biol. 17, 4792–4800
11. Patten, E. J., and DeLong, M. J. (1999) Biochem. Biophys. Res. Commun. 257, 149–155
12. John, C., Haskill, S., Mayer, L., Panja, A., and Bax, R. (1997) J. Immunol. 158, 226–234
13. Elewaut, D., Dinhoto, J. A., Kim, J. M., Truong, F., Eckmann, L., and Kagnoff, M. F. (1999) J. Immunol. 163, 1457–1466
14. Malinin, N. L., Boldin, M. P., Kovalenko, A. V., and Wallach, D. (1997) Nature 385, 540–544
15. Ghosh, S., and Karin, M. (2002) Cell 109, Suppl., 881–896
16. Kelliher, M. A., Grimm, S., Isihara, Y., Kao, F., Stanger, B. Z., and Leder, P. (1998) Immunity 8, 297–303
17. Devlin, A., Cook, A., Lin, Y., Rodriguez, Y., Kelliher, M., and Liu, Z. (2000) Immunity 12, 419–429
18. Hsu, H., Huang, J., Shu, H. B., Baichwal, V., and Goeddel, D. V. (1996) Immunity 4, 387–396
19. Lin, X., Mu, Y., Cunningham, E. T., Jr., Marco, C. B., Gelezunias, R., and Greene, W. C. (1998) Mol. Cell. Biol. 18, 5899–5907
20. Ashkenazi, A., and Dixit, V. M. (1999) Curr. Opin. Cell Biol. 11, 255–260
21. Bartek, M., and Gilmure, T. D. (1999) Oncogene 18, 6910–6924
22. Yuji, T., Waré, M., Widmann, C., Oyer, R., Russell, D., Chan, E., Ziuiz, Y., Clarke, P., Tyler, K., Oka, Y., Fanger, G. R., Henson, P., and Johnson, G. L. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7272–7277
23. Yin, L., Wu, L., Wesche, H., Arthur, C. B., White, J. M., Goeddel, D. V., and Schreiber, R. D. (2001) Science 291, 2162–2165
24. Nakano, H., Shindo, M., Sakon, S., Nishina, S., Mihara, M., Yagita, H., and Okumura, K. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3537–3542
25. Pomerantz, J. L., and Baltimore, D. (2002) Mol. Cell. 10, 693–695
26. Ryan, K. M., Ernst, M. K., Rice, N. R., and Vosud, K. H. (2000) Nature 404, 892–897

Oltipraz Activates NF-κB in QR Regulation