Transcriptional Induction of CYP1A1 by Oltipraz in Human Caco-2 Cells Is Aryl Hydrocarbon Receptor- and Calcium-dependent*

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Oltipraz, a synthetic derivative of the cruciferous vegetable product 1,2-dithiole-3-thione, is considered as one of the most potent chemoprotectants. It modulates both cytochrome P-450 (CYP) and glutathione S-transferase expression and activities in rat tissues. Its effects, however, are variable according to the enzyme, tissue, and species. We show here that, as previously found in rat lung and kidney, CYP1A1 is inducible by oltipraz in both rat intestine and Caco-2 cells, a cell line originated from a human colon adenocarcinoma. In these cells, a 50 μM oltipraz treatment increased CYP1A1 mRNA (~30-fold), protein and activity. mRNA level was augmented as early as 2 h after the beginning of treatment, suggesting a transcriptional activation, and was maximal between 8 and 12 h. Transient transfection of Caco-2 cells with constructs containing different sizes of the 5′-flanking region of the CYP1A1 gene upstream of the luciferase reporter gene showed an increase in luciferase activity in oltipraz-treated cells, which correlates with the presence of the xenobiotic responsive element (XRE). Furthermore we demonstrated that resveratrol, an antagonist of the aryl hydrocarbon (Ah) receptor, inhibited the induction of both CYP1A1 promoter activity and mRNA by oltipraz, supporting the involvement of the Ah receptor in this induction. In an attempt to further characterize the mechanism of CYP1A1 induction, we showed a rapid increase in intracellular calcium concentration upon treatment of Caco-2 cells with oltipraz. Moreover, the effect of this compound on CYP1A1 was strongly abolished in the presence of BAPTA-AM, a well known chelator of intracellular calcium, and 2-aminoethyl diphenylborate, an inhibitor of store-operated calcium channels. These results bring the first demonstration that oltipraz activates transcription of the CYP1A1 gene through the Ah receptor-XRE pathway in Caco-2 cells and that CYP1A1 induction relies upon an increase of intracellular calcium concentration.

Oltipraz, a synthetic derivative of 1,2-dithiole-3-thione, a constituent of cruciferous vegetables, is considered as one of the most promising chemopreventive agents in development, based on preclinical studies (1–5) and a recent Phase IIa clinical trial in China (6, 7). This compound was first claimed to act as a chemopreventive agent by enhancing activities of phase II enzymes such as glutathione S-transferase, UDP-glucuronosyltransferases, NAD(P)H:quinone reductase, aflatoxin B1-aldehyde reductase, and epoxide hydrolase (for a review see Ref. 8). Biochemical and genetic studies showed that induction of these detoxifying enzymes is primarily due to transcriptional activation of the genes and is regulated by an enhancer, called an antioxidant-responsive element (ARE)1 or electrophile-responsive element (9–11). NF-E2-related factor 2 (Nrf2) has been implicated as an essential component of an ARE-binding transcriptional complex (12–14), but the signal transduction pathways that relay the chemical signals to the ARE-protein complex remain to be elucidated.

More recently oltipraz was also reported to influence cytochrome P450 (CYP) expression and activities. It was found to be a potent inhibitor of both CYP1A and -2B in rat liver in vivo and in vitro (15) and of CYP1A2 and -3A4 in primary human hepatocyte cultures (16). Other studies demonstrated that oltipraz was also capable of increasing mRNA levels of CYP1A2 and CYP2B1/2 in rat liver following its transient inhibitory effect (15, 17). A similar induction of CYP1A expression and activity was observed in lung and kidney, and CYP2B1 protein was dramatically decreased via a proteasome-dependent pathway in rat lung after administration of oltipraz (18).

The CYP1A subfamily catalyzes the metabolic activation of polycyclic aromatic hydrocarbons (PAH), which generates genotoxic metabolites that bind DNA thus mediating PAH-induced carcinogenesis. The CYP1A genes do not contain any closely matching ARE sequence, indicating their transcriptional induction by oltipraz involves an ARE-independent pathway. Classically, CYP1A1/2 transcription is induced by PAH via the aryl hydrocarbon receptor (AhR). The PAH bind the cytosolic AhR ligand-binding subunit, which undergoes a translocation or activation process involving several steps: translocation in the nucleus; release of 90-kDa heat-shock proteins; and heterodimerization with a protein partner, the Ah receptor nuclear translocator (Arnt). This heterodimer interacts with a 5′-GCCGT-3′ DNA sequence, the core binding motif of the xenobiotic-responsive element (XRE), present in multiple copies upstream of the CYP1A1 gene promoter (19).

1 The abbreviations used are: ARE, antioxidant-responsive element; 2-APB, 2-aminoethyl diphenylborate; Ah, aryl hydrocarbon; AhR, aryl hydrocarbon receptor; Arnt, aryl hydrocarbon receptor nuclear translocator; BAPTA-AM, acetoxymethyl ester of 5,5′-dimethyl-bis-(o-amino-phenoxy)ethane-N,N′,N,N′′,N′-tetracetic acid; [Ca2+]i, intracellular free calcium concentration; CaMK, Ca2+/calmodulin-dependent protein kinase; CYP, cytochrome P-450; EROD, ethoxyresorufin O-deethylase; Fura-2-AM, Fura-2-acetoxyethylster; MAPK, mitogen-activated protein kinase; NrF2, NF-E2-related factor 2; PAH, polycyclic aromatic hydrocarbon; PKC, protein kinase C; TBS, Tris-buffered saline; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; XRE, xenobiotic-responsive element.
Several reports have implicated protein kinase C (PKC) in the XRE-inducible transcription of the CYP1A1 gene mediated through the AhR (20–22). Recently, Huang et al. (13) demonstrated that regulation of the ARE also involves a PKC-mediated phosphorylation of Nr2r and a mitogen-activated protein kinase (MAPK) pathway (14). Because MAPK and PKC targeting are regulated by intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) (23–25), this ubiquitous second messenger might be one of the initial events involved in gene induction mediated by oltipraz or PAH. Interestingly, it has been shown that benzof[alpha]pyrene and 7,12-dimethylbenz[a]anthracene, two known CYP1A1 inducers, increase [Ca\(^{2+}\)]\(_i\) in the human mammary epithelial cell line MCF-10A (26).

Dithiolethiones and food contaminant PAH first accumulate in the gastrointestinal tract, which expresses some CYP1A1 metabolic capacity (27), suggesting that in the presence of oltipraz, activation of these carcinogenic agents could be altered. In this study we confirmed that CYP1A1 is expressed in the rat intestine and is inducible after administration of oltipraz to rats. To further characterize the mechanism of induction of CYP1A1 by this compound, we used the Caco-2 cell line derived from a human colon carcinoma. These cells differentiate into enterocytes at confluence and exhibit a highly inducible CYP1A1 by benzimidazole or PAH derivatives (omeprazole and \(\gamma\)-naphthoflavone or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), respectively) (28, 29). We brought evidence that CYP1A1 induction by oltipraz is dependent upon both [Ca\(^{2+}\)]\(_i\) and the Ah receptor.

### MATERIALS AND METHODS

#### Chemicals—BAPTA-AM and 2-aminoethyl diphenylborate (2-APB)

were purchased from Calbiochem and EGTA from Sigma. Fura-2-ace-

#### Animal Treatment—Male Wistar rats (\(-180\) g) were fed a diet (AO4; Centre d’Elevage Janvier, Le Genest, France) supplemented with oltipraz. Oltipraz was kindly supplied by Dr. C. G. Caillard (Rhone-Poulenc Rorer, France).

#### Intracellular Calcium Measurements

Caco-2 cells, originating from a human colorectal carcinoma were obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco’s modified Eagle’s medium containing 20% fetal calf serum, 1% non-essential amino acids, 1% medium were obtained from the American Type Culture Collection (Manassas, French laws and regulation.

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#### Reporter Gene Constructs and Transient Transfection

The p1A1-FL constructs. Similar experiments were performed with a “basic” control consisting of the promoterless pGL3-luciferase construct (pGL3-baslic) and a pGL3-promoter plasmid containing the SV40 promoter upstream of the luciferase gene. Transient transfection of Caco-2 cells was performed by the TransFast method (Promega) according to manufacturer’s instructions. Briefly, 400 µl of transfection media (Dulbecco’s modified Eagle’s medium without fetal bovine serum) containing 600 ng of luciferase reporter plasmid were added to the confluent Caco-2 cells along with 15 ng of the p-SV40 DNA and 4 µl of TransFast. The plates were incubated for 2 h, and then 2 ml of Dulbecco’s modified Eagle’s medium were added at a density equivalent for another 24 h. At the time of luciferase was measured after incubated for 16 h. Dual luciferase activities (firefly and Renilla) were performed with a Promega kit as described in the manufacturer’s protocol.

### Preparation of Microsomal Fractions—Intestinal mucosa or Caco-2 cells were homogenized in 50 mM Tris-HCl buffer (pH 7.4) containing 0.25 mM sucrose and 1 mM EDTA. After removal of nuclear and mitochondrion fractions by centrifugation at 3,000 × g at 4°C, the microsomes were obtained by centrifugation at 100,000 × g for 1 h. They were dissolved in 0.1 M phosphate buffer (pH 7.4) containing 10% glycerol and stored at −80 °C until use. Protein concentration was determined according to the Bradford method (35).

### Ethoxyresorufin O-Deethylase (EROD) Activity Assay—EROD activity, supported mainly by CYP1A1, was measured on intestinal microsomes according to the method of Burke and Mayer (36). Measurement on living Caco-2 cells was performed as follows. In brief, the medium was removed after treatment, and cells were washed with 100 mM phosphate-buffered saline and then incubated with 50 µM ethoxyresorufin (Sigma) and 1.5 mM salicylamide (Sigma) at 37 °C. Kinetic reading with a spectrofluorometer was performed over a period of 30 min. Fluorescence values were converted to picomoles using a calibration curve of resorufin fluorescence, and results were expressed as pmol of resorufin/mg of total protein/min.

### Western Blot Immunnoassays—Caco-2 microsomal proteins were dissolved in 10% SDS, 1% \(\beta\)-mercaptoethanol, 10 mM Tris-HCl pH 6.8, and 20% glycerol. Twenty micrograms of protein were electrophoresed on a 15% polyacrylamide slab gel and electrophroblotted overnight onto Hybond enhanced chemiluminescence nitrocellulose membranes (ECL, Amer-

### Intracellular Calcium Measurements—Caco-2 cells were cultured on 12-mm culture inserts (0.4 µm pore size, polycarbonate, Transwell, Costar) and incubated with 2.5 mM Fura-2-AM for 15 min at 37°C in Hepes-buffered medium (10 mM Hepes, 134.8 mM NaCl, 4.7 mM KCl, 1 g mitochondrial fractions by centrifugation at 3,000 × g for 1 h. Peroxidase activity was detected using ECL Western blotting detection system (Amersham Biosciences).

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RESULTS

Effect of Oltipraz on CYP1A1 Expression and Activity in Rat Gut—In a first set of experiments we investigated the expression of CYP1A1 in rat intestine, one of the first tissues in contact with oltipraz after administration by oral route. To differentiate the different parts of the gastrointestinal tract, we measured CYP1A1 mRNA levels in the duodenum, jejunum, ileum, and colon of control and oltipraz-treated rats. After 72 h of treatment with oltipraz, CYP1A1 mRNA levels were increased mainly in the duodenum and jejunum when compared with control rats (Fig. 1A). CYP1A1 activity, measured by the deethylation of ethoxyresorufin (EROD), was also augmented in the duodenum and jejunum (Fig. 1B), although a significant increase was observed only in the jejunum (9-fold; p < 0.05).

Effect of Oltipraz on CYP1A1 Expression and Activity in Human Caco-2 Cells—To determine whether CYP1A1 is also induced in human cells by oltipraz, we used human Caco-2 cells, which are able to differentiate after ~21 days of confluency into cells expressing enterocyte characteristics. The differentiated state of the cells was confirmed by measuring the sucrase-isomaltase mRNA levels (data not shown). Twenty-one days after confluency, the cells were treated with 50 μM oltipraz for 2, 4, 8, 12, 18, and 24 h, and CYP1A1 mRNA and protein levels and activity were determined by Northern blot, Western blot, and measurement of EROD activity, respectively (Fig. 2A). In control cells, CYP1A1 transcripts and proteins were not detected. However, an increase of CYP1A1 mRNA level as early as 2 h after the beginning of oltipraz treatment was observed, whereas EROD activity and protein level were augmented after 4 h of treatment. The maximum of mRNA induction was obtained between 8 and 12 h of treatment. Treatment of Caco-2 cells with benzo[a]pyrene, a PAH, elicited the same pattern of CYP1A1 mRNA induction (Fig. 2B).

Effect of Oltipraz on CYP1A1 Transcriptional Activity in Human Caco-2 Cells—The rapid induction of CYP1A1 mRNA in oltipraz-treated cells suggested the involvement of a transcriptional mechanism. To test this hypothesis, we performed transient transfections with constructs containing the CYP1A1 gene 5′-flanking region upstream of the luciferase gene in order to measure the transcriptional activity of CYP1A1 in Caco-2 cells in the absence or presence of oltipraz. As shown in Fig. 3, neither pGL3-basic nor pGL3-promoter luciferase activities were affected after oltipraz treatment. In contrast, transfection of pA1-FL(-1566) construct, which contains 1566 bp of the CYP1A1 gene 5′-flanking region upstream of the luciferase gene, resulted in a 5-fold increase of the luciferase activity in oltipraz-treated cells. CYP1A1 promoter activity was still induced by oltipraz when CYP1A1 deletion constructs (from −1566 to −1300 of the CYP1A1 promoter) were used. In contrast, there was no significant increase of luciferase activity when pA1-FL(−800) or pA1-FL(−340) constructs were transfected. Interestingly, five XREs, which are known to be involved in the CYP1A1 regulation by PAH, are present in the pA1-FL(−1566), only one in the pA1-FL(−800), and none in the pA1-FL(−340) construct (19). Because oltipraz induction of CYP1A1 promoter activity seems to be correlated to the presence of XRE in the CYP1A1 DNA fragment subcloned upstream of the luciferase gene, Caco-2 cell transfection was performed with a construct containing a luciferase reporter gene driven by three XREs (pGL3-XRE3). Results from this experiment showed that the XRE-driven luciferase activity is inducible by oltipraz (2.5-fold, p < 0.05).

In cells treated by PAH or derivatives such as dioxin, the
variations in the presence of oltipraz, representative of [Ca2\(^{2+}\)] fluorescence ratio (F340/F380) were observed. F340/F380 ratio changes in concentrations of oltipraz, significant changes in the Fura-2 test) was performed by comparing oltipraz-treated and control cells (*, \(p < 0.05\); **, \(p < 0.01\); ***, \(p < 0.001\)).

Examples performed in triplicate is shown. The values are expressed as means ± S.D. of three measurements. Statistical analysis (Student’s \(t\) test) was performed by comparing oltipraz-treated and control cells (*, \(p < 0.05\); **, \(p < 0.01\); ***, \(p < 0.001\)).

Effects of Oltipraz on Intracellular Calcium Concentration—To further determine the mechanism involved in CYP1A1 induction by oltipraz, we studied upstream events that might occur at the cellular level and more particularly the [Ca2\(^{2+}\)]. In a first set of experiments we measured the [Ca2\(^{2+}\)], in Caco-2 cells in the absence or presence of oltipraz by using cell loaded-Fura-2-AM as a Ca2\(^{2+}\)-sensitive fluorescent probe. The intracellular Fura-2 fluorescence ratios recorded in the presence or absence of oltipraz were compared, knowing that any significant change in these ratios are suggestive of a significant change in [Ca2\(^{2+}\)].

Following short-term incubations of Caco-2 cells with different concentrations of oltipraz, significant changes in the Fura-2 fluorescence ratio (F340/F380) were observed. F340/F380 ratio variations in the presence of oltipraz, representative of [Ca2\(^{2+}\)], variations, are illustrated by the recordings given in the left panel of Fig. 5A. An increase of the ratio was already observed with 5 \(\mu\)M of oltipraz, which became more pronounced with increasing concentrations of oltipraz (25, 50, and 100 \(\mu\)M). The delta ratio, corresponding to the difference between the F340/F380 ratios measured in cells prior to and following oltipraz treatment, respectively, showed a correlation between oltipraz concentration and [Ca2\(^{2+}\)]. (Fig. 5A, right panel).

To determine whether the increase of [Ca2\(^{2+}\)], following oltipraz treatment was the consequence of an extracellular calcium entry, the cells were maintained in calcium-free medium supplemented with 10 mM EGTA (an extracellular calcium chelator) and treated with oltipraz. Under these conditions, the [Ca2\(^{2+}\)] increase was lowered dramatically in the calcium-free medium when compared with the [Ca2\(^{2+}\)], increase obtained with cells maintained in a calcium-containing medium (Fig. 5B).
The increase of \([\text{Ca}^{2+}]\) was observed very rapidly after the addition of oltipraz to the culture medium, reaching a maximum within a few minutes. To estimate whether this effect was transient, Caco-2 cells were incubated with 50 \(\mu\text{M}\) oltipraz for 15 min and 1, 2, 6, 12, and 24 h. As shown in Fig. 6, the oltipraz-induced increase of \([\text{Ca}^{2+}]\) was significant only at 15 min and at 1 h of treatment. No significant elevations of \([\text{Ca}^{2+}]\) were observed with longer times of treatment (2, 6, 12, and 24 h). These results demonstrated that the oltipraz-induced \([\text{Ca}^{2+}]\) was a transient phenomenon.

**Is CYP1A1 Induction by Oltipraz Mediated by an Increase of \([\text{Ca}^{2+}]\)?**—To determine whether there is a link between the early increase of \([\text{Ca}^{2+}]\), and the induction of CYP1A1 in Caco-2 cells treated with oltipraz, we used an intracellular chelator of calcium, BAPTA-AM. One hour prior to oltipraz treatment, the cells were incubated with 25 \(\mu\text{M}\) BAPTA-AM. Fig. 5C demonstrates the effectiveness of this chelator to inhibit the increase of \([\text{Ca}^{2+}]\) in cells treated with oltipraz. In the next set of experiments, we measured CYP1A1 activity and mRNA level in control and oltipraz-treated cells in the absence or presence of BAPTA-AM, added 1 h prior to oltipraz treatment. The results shown in Fig. 7A demonstrate that 12.5–50 \(\mu\text{M}\) intracellular calcium chelator partially abolished the oltipraz induction of CYP1A1. This effect was also observed at the apoprotein level (data not shown). Interestingly, the inhibitory effect of BAPTA-AM on oltipraz-CYP1A1 induction was less effective
Human CYP1A1 Induction by Oltipraz in Caco-2 Cells

CYP1A1 appears to be among the most sensitive of the CYPs. CYP1A1 is expressed both in the liver and extrahepatic tissues, such as the lung, intestine, and skin, where it is involved in the bioactivation of a number of carcinogens such as PAH as well as other toxicants.

The pleiotropic effects of oltipraz, a promising chemopreventive agent, are now well recognized, and recent evidence has been brought that this compound can induce both phase I and phase II enzymes in extrahepatic tissues. Although differences have been observed regarding the response of phase I and phase II enzymes between rat tissues, CYP1A1 appears to be among the most sensitive of the CYPs. CYP1A1 and CYP1A2, which are expressed, respectively, mostly in nonhepatic and hepatic tissues, are involved in the biotransformation of a number of carcinogens such as PAH as well as other toxicants. The results reported here show that oltipraz is also an effective inducer of CYP1A1 activity in Caco-2 cells.

Effect of the Intracellular Calcium Chelator, BAPTA-AM, on Oltipraz Induction of CYP1A1 mRNA Expression and Activity in Caco-2 Cells. Caco-2 cells were treated or not with oltipraz during 12 h in the presence or absence of 12.5, 25, or 50 μM BAPTA-AM (added 1 h prior oltipraz). CYP1A1 activity was measured by the deethylation of EROD in control or treated Caco-2 cells. Statistical analysis (Student's t test) was performed by comparing oltipraz-treated cells with oltipraz/BAPTA-AM-treated cells (**, p < 0.01; *, p < 0.05). Total RNA was isolated from control and oltipraz-treated cells in the presence or absence of 12.5, 25, or 50 μM BAPTA-AM and subjected to Northern blot analysis. Hybridization of the same filters was performed successively with a CYP1A1-specific cDNA probe and an 18 S oligonucleotide probe used as controls. CYP1A1 activity was measured by the deethylation of EROD in Caco-2 cells treated or not for 12 h with oltipraz in the presence or absence of 50 μM BAPTA-AM. BAPTA-AM was added 60 min before (Before) or 30, 60, and 240 min after (After) the addition of oltipraz. Statistical analysis (Student's t test) was performed by comparing oltipraz-treated cells with oltipraz/BAPTA-AM-treated cells (**, p < 0.01; *, p < 0.05). Total RNA was isolated from control and oltipraz-treated cells in the presence or absence of BAPTA-AM (added 1 h prior oltipraz). CYP1A1 activity was measured by the deethylation of EROD in control or oltipraz-treated (12 h) Caco-2 cells. The cells were pretreated during 30 min or not with 50, 75, or 100 μM 2-APB. Statistical analysis (Student's t test) was performed by comparing oltipraz-treated cells with oltipraz/2-APB-treated cells (***, p < 0.001; **, p < 0.01). Total RNA was isolated from control and oltipraz-treated cells in the presence or absence of 2-APB and subjected to Northern blot analysis. Hybridization of the same filters was performed successively with a CYP1A1-specific cDNA probe and an 18 S oligonucleotide probe used as controls.

When the calcium chelator was added 30, 60, and 240 min after oltipraz (Fig. 7B), thus indicating that the rapid, transient increase of [Ca2+]i elicited upon addition of oltipraz (between 0 and 1 h) initiates the CYP1A1 induction mechanism. The oltipraz induction of luciferase activity measured after transient transfection of p1A1-FL or pGL3-XRE3 was abolished in the presence of 50 μM BAPTA-AM (Fig. 8). This finding also strongly supports the involvement of intracellular calcium in the oltipraz-mediated induction of CYP1A1 in Caco-2 cells.

To determine the mechanism involved in the increase of [Ca2+]i, following oltipraz treatment, we used verapamil and nifedipine, two inhibitors of voltage-dependent L-type calcium channels, and 2-APB, a blocker of store-operated Ca2+ channels. Treatment of Caco-2 cells with nifedipine or verapamil did not affect oltipraz-CYP1A1 induction (data not shown). On the other hand, 2-APB inhibited CYP1A1 induction in a dose-dependent manner (Fig. 9) and was found to prevent oltipraz-induced [Ca2+]i increase (data not shown). These results strongly suggest that calcium entering through store-operated Ca2+ channels is essential for CYP1A1 induction in Caco-2 cells treated with oltipraz.

FIG.7. Effect of the intracellular calcium chelator, BAPTA-AM, on oltipraz induction of CYP1A1 mRNA expression and activity in Caco-2 cells. Caco-2 cells were treated or not with oltipraz during 12 h in the presence or absence of 12.5, 25, or 50 μM BAPTA-AM (added 1 h prior oltipraz). CYP1A1 activity was measured by the deethylation of EROD in control or treated Caco-2 cells. Statistical analysis (Student's t test) was performed by comparing oltipraz-treated cells with oltipraz/BAPTA-AM-treated cells (**, p < 0.01; *, p < 0.05). Total RNA was isolated from control and oltipraz-treated cells in the presence or absence of 12.5, 25, or 50 μM BAPTA-AM and subjected to Northern blot analysis. Hybridization of the same filters was performed successively with a CYP1A1-specific cDNA probe and an 18 S oligonucleotide probe used as controls. A, CYP1A1 activity was measured by the deethylation of EROD in Caco-2 cells treated or not for 12 h with oltipraz in the presence or absence of 50 μM BAPTA-AM. BAPTA-AM was added 60 min before (Before) or 30, 60, and 240 min after (After) the addition of oltipraz. Statistical analysis (Student's t test) was performed by comparing oltipraz-treated cells with oltipraz/BAPTA-AM-treated cells (**, p < 0.01; *, p < 0.05). Total RNA was isolated from control and oltipraz-treated cells in the presence or absence of BAPTA-AM (added 1 h prior oltipraz). CYP1A1 activity was measured by the deethylation of EROD in control or oltipraz-treated (12 h) Caco-2 cells. The cells were pretreated during 30 min or not with 50, 75, or 100 μM 2-APB. Statistical analysis (Student's t test) was performed by comparing oltipraz-treated cells with oltipraz/2-APB-treated cells (***, p < 0.001; **, p < 0.01). Total RNA was isolated from control and oltipraz-treated cells in the presence or absence of 2-APB and subjected to Northern blot analysis. Hybridization of the same filters was performed successively with a CYP1A1-specific cDNA probe and an 18 S oligonucleotide probe used as controls.

FIG.9. Effect of 2-APB on induction of CYP1A1 mRNA and activity in Caco-2 cells. CYP1A1 activity was measured by the deethylation of EROD in control or oltipraz-treated (12 h) Caco-2 cells. The cells were pretreated during 30 min or not with 50, 75, or 100 μM 2-APB. Statistical analysis (Student's t test) was performed by comparing oltipraz-treated cells with oltipraz/2-APB-treated cells (***, p < 0.001; **, p < 0.01). Total RNA was isolated from control and oltipraz-treated cells in the presence or absence of 2-APB and subjected to Northern blot analysis. Hybridization of the same filters was performed successively with a CYP1A1-specific cDNA probe and an 18 S oligonucleotide probe used as controls.
inducer of CYP1A1 in the intestine and, using human Caco-2 cells, that its induction is calcium- and Ah receptor-dependent.

To our knowledge, this is the first demonstration that oltipraz dramatically increases CYP1A1 in both rat intestine and human Caco-2 cells. Moreover, the data presented here strongly indicate that this induction occurs through the XRE and that oltipraz and PAH inducers utilize the same AhR-mediated signal transduction pathway to regulate the expression of CYP1A1. First, a comparative analysis of CYP1A1 mRNA levels shows the same pattern of induction in the presence of oltipraz or benzo[a]pyrene (starting after 2 h with a maximum induction between 8 and 12 h). Second, Caco-2 cells are known to possess a functional AhR (29). Third, the expression of CYP1A1 mRNA was superinduced when oltipraz was used in the presence of a protein synthesis inhibitor, cycloheximide (data not shown). Fourth, use of luciferase reporter constructs demonstrates that oltipraz is a potent inducer of both the XRE-containing 5'-flanking region of CYP1A1 gene and XRE-driven gene expressions. Fifth, resveratrol (an antagonist of AhR) inhibits the oltipraz increase of both CYP1A1 mRNA level and p1A1-Fl- or pGL3-XRE3-luciferase activities. Finally, oltipraz shares several structural features with CYP1A1/2 classical inducers such as 3-methylcholanthrene, TCDD, and β-naphthoflavone; it is hydrophobic, planar, and of polycyclic structure. Although its behavior is quite similar to that of PAH, it cannot be excluded that oltipraz (or one of its metabolites) is an activator of AhR without binding to it, as already shown for other compounds such as omeprazole (42, 43).

Previous reports and the present study clearly indicate that oltipraz is able to increase transcriptional activity of phase II enzymes and CYP1A1 through the binding of transcriptional factors to the ARE and XRE sequences, respectively. However, early cellular events occurring upon cell exposure to oltipraz and leading to this transcriptional activation are still unknown and remain to be described. In an attempt to determine whether calcium, which is a well established transcriptional regulator, is involved in CYP1A1 induction, we looked for an effect of oltipraz on intracellular calcium level in Caco-2 cells. Our results clearly demonstrate that oltipraz induces a rapid and transient increase of [Ca2+]i in these cells, which mediates oltipraz induction of CYP1A1. Increases in intracellular calcium in most cell types are mediated by either the release of calcium from the endoplasmic reticulum via inositol 1,4,5-triphosphate or an influx via plasma membrane Ca2+ channels (voltage-dependent calcium channels, receptor-operated calcium channels, and/or store-operated Ca2+ channels) by both. In the present case, the lack of [Ca2+]i increase following the addition of oltipraz in a calcium-free medium (containing EGTA) strongly supports the involvement of plasma membrane calcium channels. This was confirmed by using 2-APB, an inhibitor of store-operated Ca2+ channels, which inhibits oltipraz-CYP1A1 induction in a dose-dependent manner. Store-operated Ca2+ channels are defined as plasma membrane Ca2+ channels, in which activation is mediated by a decrease in Ca2+ in the endoplasmic reticulum (44). These channels are thought to be responsible for replenishing Ca2+ lost from the endoplasmic reticulum via membrane Ca2+/Mg2+-ATPase. Thus, the addition of oltipraz in the cell culture medium rapidly increases [Ca2+]i via an entry of this ion species through store-operated channels, which is likely to be activated following an effect on the endoplasmic reticulum. Our results also support the hypothesis that this rapid increase of [Ca2+]i is the initial event leading to the induction of CYP1A1 by oltipraz. Interestingly, Marc et al. (45) have brought evidence for the involvement of Ca2+ in the induction of mouse Cyp2b9/10 by phenobarbital, a compound that also induces CYP1A1 in rat (46) and CYP1A2 in rainbow trout hepatocytes (47). Moreover, TCDD, benzo[a]pyrene, and 7,12-dimethylbenz(a)anthracene, three known CYP1A1 inducers, have been reported to increase [Ca2+]i in the human mammary epithelial cell line MCF-10A (26, 48). Therefore, intracellular Ca2+ might be a key factor in the induction of CYP1A1 by various compounds. This hypothesis is reinforced by experiments carried out with Caco-2 cells treated with BAPTA-AM or 2-APB, which also partially inhibit CYP1A1 induction by TCDD, benzo[a]pyrene, or 3-methylcholanthrene (data not shown).

Several studies have demonstrated that changes in free intracellular Ca2+ are rapidly transformed into changes in the activity of several kinases including protein kinase A, PKC, MAPKs (extracellular signal-regulated kinase and p38), Ca2+/calmodulin-dependent protein kinase (CaMK), and CaMKK (23, 49). Previous investigations revealed that DNA binding by human and mouse AhR-Arnt heterodimers requires phosphorylation of both proteins, whereas formation of AhR-Arnt heterodimers requires phosphorylation of Arnt only (20, 22). Moreover, Long et al. (21) showed that a PKC-mediated event is required for the AhR to form a functional transcriptional complex that leads to trans-activation. Thus, in the process of CYP1A1 induction by oltipraz, [Ca2+]i might be a determinant step in the activation of PKC and hence the phosphorylation of AhR. Interestingly, recent findings indicate an important role for both PKC and MAPK pathways in the induction of ARE-dependent phase II detoxifying enzymes, with Nrf2 being phosphorylated by PKC (13, 14). Consequently, it could be postulated that the increases in both CYP1A1 and phase II enzymes in the presence of oltipraz are dependent on a common initial signal, which is the oltipraz-induced entry of calcium inside the cells. This has yet to be established regarding phase II enzymes. Thus, the entry of extracellular calcium inside the cells through store-operated Ca2+ channels might be the initial event leading to the activation of the AhR receptor and its subsequent binding to the XRE sequence.

Although oltipraz exhibits a chemopreventive effect during different stages of experimental colon carcinogenesis induced by azoxymethane in rats (4), compounds that induce CYP1A1 are regarded as potentially harmful because of the property of this CYP to activate carcinogenic PAH. Furthermore, rat intestinal conjugation enzymes have been demonstrated to be less susceptible to induction by oltipraz than their hepatic counterparts (50, 51). In this context, the ability of oltipraz to induce CYP1A1 in intestinal cells raises the critical question of whether, in some cases, oltipraz might potentiate the deleterious effects of a particular compound to which an individual is also exposed.

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Transcriptional Induction of \textit{CYP1A1} by Oltipraz in Human Caco-2 Cells Is Aryl Hydrocarbon Receptor- and Calcium-dependent

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