Oxidized Metabolites of Oltipraz Exert Cytoprotective Effects against Arachidonic Acid through AMPK-dependent Cellular Antioxidant Effect and Mitochondrial Protection

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ABBREVIATIONS: AA, arachidonic acid; ACC, acetyl-CoA carboxylase; AICAR, 5-aminooimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, AMP-activated protein kinase; C/EBP, CCAAT/enhancer binding protein; cyt c, cytochrome c; DCFH-DA, 2',7'-dichlorofluorescin diacetate; DMEM, Dulbecco's modified Eagle's medium; DN-AMPK, dominant negative mutant of AMPK; FBS, fetal bovine serum; GSH, glutathione; GST, glutathione S-transferase; MMP, mitochondrial membrane potential; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; NAC, N-acetyl-L-cysteine; Nrf2, NF-E2-related factor-2; PARP, poly(ADP-ribose)polymerase; PPD, pyrrolopyrazine thione; Rh123, rhodamine 123; ROS, reactive oxygen species
ABSTRACT:
Oltipraz protects cells from chemical-induced carcinogenesis partly due to phase 2 enzyme induction. Certain oltipraz metabolites also induce phase 2 enzymes. This study investigated the cytoprotective effects of the oxidized metabolites of oltipraz against arachidonic acid (AA), a proinflammatory fatty acid that causes cellular reactive oxygen species (ROS) production and mitochondrial impairment, and the mechanistic basis of their action in HepG2 cells. Treatment with M1 (4-methyl-5-(pyrazin-2-yl)-3H-1,2-dithiol-3-one) or M2 (7-methyl-6,8-bis(methylthio)H-pyrrolo[1,2-a]pyrazine), but not M3 (7-methyl-8-(methylsulfinyl)-6-(methylthio)H-pyrrolo[1,2-a]pyrazine) or M4 (7-methyl-6,8-bis(methylsulfinyl)H-pyrrolo[1,2-a]pyrazine), enabled cells to protect against AA-induced apoptosis. M1 and M2 treatment protected cells from ROS produced by AA, and inhibited AA-induced glutathione (GSH) depletion. Moreover, both M1 and M2 effectively inhibited mitochondrial dysfunction induced by AA although M2 alone slightly elicited it at a relatively high concentration. M1 and M2 activated AMP-activated protein kinase (AMPK), but M3 and M4 failed to do so. AMPK activation by M1 and M2 contributed to cell survival against AA through a decrease in cellular ROS production and prevention of mitochondrial dysfunction, as shown by the reversal of the metabolites’ restoration of mitochondrial membrane potential by compound C treatment or overexpression of a dominant negative mutant AMPK. Consistently, AICAR, an AMPK activator, also had a cytoprotective and antioxidant effect against AA. Our results demonstrate that, of oltipraz’s major metabolites, M1 and M2 are capable of protecting cells from AA-induced ROS production and mitochondrial dysfunction, which may be associated with AMPK activation.
Introduction

Oltipraz [4-Methyl-5-(2-pyrazinyl)-1,2-dithiole-3-thione] has been widely studied as a cancer chemopreventive agent (Kang et al., 2003; Bolton et al., 1993; Jacobson et al., 1997; Wang et al., 1999). Comprehensive mechanistic studies indicated that the cancer chemopreventive effects of oltipraz might be associated with the enhancement of NF-E2-related factor-2 (Nrf2)'s antioxidant response element binding activity and the consequent changes in target gene transactivation (e.g., phase II antioxidant enzymes) (Kensler, 1997; Ramos-Gomez et al., 2001). It has also been shown that oltipraz enhances the induction of the glutathione S-transferase (GST) A2 gene via CCAAT/enhancer binding protein-β (C/EBPβ) activation (Kang et al., 2003). Moreover, oltipraz has a therapeutic effect on the cirrhotic liver (Kang et al., 2002; Cho et al., 2006). Recently, we also found that oltipraz prevents insulin resistance induced by tumor necrosis factor-α (TNFα), a cytokine known to promote production of reactive oxygen species (ROS), as a consequence of AMP-activated protein kinase (AMPK) activation (Bae et al., 2007). In addition, oltipraz exerts a cytoprotective effect against arachidonic acid (AA) and/or iron via AMPK (Shin and Kim, 2009).

Oltipraz tends to accumulate in organs, especially liver, large intestine and fat due to its lipophilicity (Bae et al., 2004). At high concentrations, oltipraz residence time in the body is prolonged, which may increase the production of its oxidized metabolites via extensive metabolism by the two major pathways common to various mammalian species: first, oxidative desulfuration of the thione to yield M1, which does not appear to be metabolized further; and second, desulfuration, methylation and molecular rearrangement to yield M2, which can be metabolized to other oxidized forms, M3 and M4 (O’Dwyer et al., 1997; Bieder et al., 1983). It has been shown that M1 was active in inducing NAD(P)H:quinone oxidoreductase (DT-diaphorase) in adenocarcinoma cells (O’Dwyer et al., 1997). Both M1 and M2 are active in inducing the GSTA2 gene, whereas M3 and M4 are inactive. In H4IIE cells, M1 induced GSTA2 via C/EBPβ activation, while M2 did so by activating Nrf2 as well as C/EBPβ (Ko et al., 2006).

Increased production of ROS may induce oxidative stress, which is implicated in cellular injury, resulting in ATP and NAD depletion, DNA and protein damage, and glutathione (GSH) depletion.
Excess ROS may promote the inflammatory process, cell death, and fibrosis (Browning and Horton, 2004). It is well recognized that oxidative stress may produce alterations in membrane phospholipids. Oxidative modification of fatty acids within phospholipids may affect the process of cell signaling. In response to ROS and proinflammatory cytokines, rearranged membrane phospholipids activate phospholipase A2 (PLA2) (Cummings et al., 2000; Balboa and Balsinde, 2006). In cells exposed to oxidative stress, PLA2 increases the release of AA, a biologically active proinflammatory mediator, from phospholipids in the cell membrane (Balboa and Balsinde, 2006).

AA is an ω-6 polyunsaturated fatty acid that mediates oxidative stress and inflammation. High ω-6/ω-3 fatty acid ratios are found in patients with certain diseases (e.g., cardiovascular disease, cancer, osteoporosis, and inflammatory and autoimmune diseases) (reviewed by Simopoulos, 2006). AA produced in large quantities by oxidative stress is utilized for the production of pro-apoptotic prostaglandins or leukotrienes (Neale et al., 1988; Woo et al., 2002). For example, AA directly activates sphingomyelinase which leads to the breakdown of sphingomyelin and the generation of ceramide, thereby initiating apoptotic cell death (Jayadev et al., 1994). Furthermore, AA releases calcium from intracellular stores, and increases mitochondrial uptake of calcium during apoptosis (Maia et al., 2006). Moreover, other studies have indicated that AA promotes cellular ROS production, causes a decrease in mitochondrial respiratory activity, and thereby induces permeability transition change (Cocco et al., 1999; Scorrano et al., 2001).

In view of the importance of antioxidant gene induction and the potential cytoprotective effect by oltipraz and its oxidized metabolites, this study investigated whether oltipraz’s oxidized metabolites protect cells from cellular ROS production and mitochondrial dysfunction induced by a proapoptotic inflammatory fatty acid. Here, we report that M1 and M2, but not M3 and M4, exert cytoprotective effects against AA through AMPK-dependent inhibition of cellular hydrogen peroxide production and of subsequent mitochondrial impairment.
Materials and Methods

Materials. MitoSOX was supplied from Molecular Probes (Carlsbad, CA). Anti-procaspase-3, anti-phospho-acetyl-CoA carboxylase (ACC) and anti-phospho-AMPK antibodies were purchased from Cell Signaling Technology (Beverly, MA). Antibodies directed against poly(ADP-ribose)polymerase (PARP), Bcl-xL and AMPK were provided from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgGs were obtained from Zymed Laboratories (San Francisco, CA). Compound C was supplied from Calbiochem (Darmstadt, Germany). DeadEnd™ Colorimetric TUNEL System was obtained from Promega (Madison, WI). Arachidonic acid, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), rhodamine123 (Rh123), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), anti-β-actin antibody, Trolox, N-acetyl-L-cysteine (NAC), and other reagents were provided from Sigma (St. Louis, MO).

Chemical Synthesis. Oltipraz and its metabolites (Fig. 1A) were synthesized at the CJ Central Laboratories (Ichon city, Korea), as described previously (Ko et al., 2006).

Cell Culture. HepG2 cells, a human hepatocyte-derived cell line, were supplied from ATCC (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 50 units/ml penicillin, and 50 μg/ml streptomycin at 37°C in humidified atmosphere with 5% CO₂. For all experiments, cells (1×10⁶) were plated in 6-well plates for 2–3 days (i.e. 80% confluency) and serum-starved for 24 h. The cells were incubated with 10 μM AA for the indicated time periods. To assess the effects of oltipraz and its metabolites, the cells were pretreated with 30 μM oltipraz or 3–30 μM of its metabolites for 30 min and then further incubated in combination with AA.

MTT Cell Viability Assay. HepG2 cells were plated at a density of 1×10⁵ cells per well in a 48-well plate to measure cytotoxicity. After treatment, viable cells were stained with MTT (0.25 mg/ml, 2 h). The media were then removed and formazan crystals produced in the wells were dissolved by addition of 300 μl dimethylsulfoxide. Absorbance was measured at 540 nm using an ELISA microplate reader (Tecan, Research Triangle Park, NC). Cell viability was defined relative to untreated
control [i.e. viability (% control) = 100 × (absorbance of treated sample)/(absorbance of control)].

**TUNEL Assay.** The TUNEL assay was performed using a commercially available kit, the DeadEnd™ Colorimetric TUNEL System, according to the manufacturer’s instructions. HepG2 cells were fixed with 10% buffered formalin in PBS at room temperature for 30 min and permeabilized with 0.2% Triton X-100 for 5 min. After washing with PBS, each sample was incubated with biotinylated nucleotide and terminal deoxynucleotidyltransferase in 100 µl of equilibration buffer at 37°C for 1 h. The reaction was stopped by immersing the samples in 2× saline sodium citrate buffer for 15 min. The activities of endogenous peroxidases were blocked by immersing the samples in 0.3% H₂O₂ for 5 min. The samples were treated with 100 µl of horseradish peroxidase-labeled streptavidin solution (1:500) and incubated for 30 min. Finally, the samples were developed using the diaminobenzidine substrate, chromogen, H₂O₂ and diaminobenzidine for 10 min. The samples were washed and examined under light microscope (200×). Cell counts were repeated four times, and the percentage from each was calculated.

**Immunoblot Analysis.** Cell lysates were prepared according to previously published methods (Kang et al., 2003). Briefly, the cells were centrifuged at 3,000 g for 3 min and allowed to swell after the addition of lysis buffer. The lysates were centrifuged at 10,000 g for 10 min to obtain supernatants and stored at −70°C until use. Immunoblot analysis was performed according to previously published procedures (Bae et al., 2007). Protein bands of interest were developed using the ECL chemiluminescence system (Amersham, Buckinghamshire, UK). Equal loading of protein was verified by immunoblotting for β-actin.

**Determination of Reduced GSH.** Reduced GSH in the cells was quantified using a commercially available GSH determination kit (Oxis International, Portland, OR, USA) (Kim et al., 2006). Briefly, the GSH-400 method was based on a two-step chemical reaction. The first step led to the formation of substitution products (thioethers) between 4-chloro-1-methyl-7-trifluoromethyl-quinolinum methylsulfate and all mercaptans that had been comprised in the sample. The second step involved a β-elimination reaction in alkaline conditions. This reaction was mediated by 30% NaOH which specifically transformed the substituted product (thioether) obtained with GSH into a chromophoric
Flow Cytometric Analysis of Mitochondrial Membrane Potential (MMP). MMP was measured with Rh123, a membrane-permeable cationic fluorescent dye. The cells were treated as specified, stained with 0.05 μg/ml Rh123 for 1 h, and harvested by trypsinization. After washing with PBS containing 1% FBS, the change in MMP was monitored using a BD FACSCalibur flow cytometer (San Jose, CA). In each analysis, 15,000 events were recorded.

Measurement of Hydrogen Peroxide Production. DCFH-DA is a cell-permeable non-fluorescent probe which is cleaved by intracellular esterases and turns into a highly fluorescent dichlorofluorescein upon reaction with hydrogen peroxide. After treatments of HepG2 cells, the cells were stained with 10 μM DCFH-DA for 30 min at 37°C. Hydrogen peroxide generation was determined by the increase in dichlorofluorescein fluorescence. The fluorescence intensity in the cells was measured using a BD FACSCalibur flow cytometer (San Jose, CA). In each analysis, 10,000 events were recorded.

Measurement of Mitochondrial ROS. MitoSOX is a live-cell-permeable and mitochondrial-localizing superoxide indicator. After treatment of HepG2 cells with AA, the cells were stained with 5 μM MitoSOX for 10 min at 37°C. The fluorescence intensity in the cells was measured using a BD FACSCalibur flow cytometer (San Jose, CA). In each analysis, 10,000 events were recorded.

Recombinant Adenoviral DN-AMPKα Construct. The plasmid encoding a dominant negative mutant of AMPKα (D157A; DN-AMPKα) was kindly provided from Dr. J. Ha (Kyung Hee University, Korea) (Bae et al., 2007). To generate recombinant adenovirus which expresses DN-AMPKα, the construct was subcoloned into an attL-containing shuttle plasmid, pENTR-BHRNX (Newgex, Seoul, Korea). Recombinant adenoviral DN-AMPKα was constructed and generated by using the pAd/CMV/V5-DEST gateway plasmid (Invitrogen, Carlsbad, CA). HepG2 cells were infected with adenovirus diluted in DMEM containing 10% FBS at a multiplicity of infection (MOI) of 50 and incubated for 12 h. After removal of the viral suspension, the cells were further incubated with DMEM containing 10% FBS for 2 days followed by treatment with reagent as indicated in the Figure Legends. Adenoviral LacZ (Ad-LacZ) was used as an infection control. Efficiency of infection was consistently
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>90% with this method.

**Data Analysis.** Scanning densitometry was performed with Image Scan & Analysis System (Alpha-Innotech Corporation, San Leandro, CA). One-way analysis of variance (ANOVA) tests were used to assess the significance of differences among treatment groups. For each statistically significant effect of treatment, the Newman-Keuls test was used for comparisons between multiple group means. The data were expressed as means ±S.E. The criterion for statistical significance was set at $p<0.05$ or $p<0.01$. 
Results

Inhibition of AA-induced Cell Death by Oltipraz’s Metabolites. First, we determined whether oltipraz’s major metabolites were capable of inhibiting apoptosis induced by AA. In the previous study, AA significantly induced cell death at 10 μM or above (Shin and Kim, 2009). In each of the experiments in this paper, 10 μM AA was used. Light microscopic analysis indicated the apoptotic morphological changes of cells treated with AA for 24 h (Fig. 1B, upper). Pretreatment with M1 or M2 (30 μM each) inhibited apoptotic morphological changes induced by AA, whereas both M3 and M4 failed to do so. The MTT assay indicated that pretreatment with M1 or M2, but not M3 or M4, protected cells from the injury induced by AA in a concentration-dependent manner (Fig. 1B, lower). The maximal cytoprotective effect was observed at 30 μM of M1 or M2. M2 also had a comparable cytoprotective effect at 10 μM. To comparatively evaluate the effects of the metabolites, the same 30 μM concentration was chosen in subsequent experiments. We further determined whether the metabolites of oltipraz prevented the ability of AA to induce apoptosis by TUNEL assay (Fig. 1C). AA increased TUNEL-positive cells (dark brown staining), which was prevented by pretreatment with M1 or M2, but not M3 or M4. To verify the induction of apoptosis by AA, cell lysates were immunoblotted for marker proteins associated with apoptosis (Fig. 1D). We found that AA treatment caused PARP cleavage, caspase-3 activation (shown as a decrease in the level of procaspase-3), and decreased the level of Bcl-xL, all of which confirmed apoptosis. The results of immunoblot analyses showing that M1 or M2 treatment completely inhibited alterations in the levels of proteins associated with apoptosis, verified the cytoprotective effect.

Inhibition of AA-induced Oxidative Stress by M1 or M2. DCFH-DA is relatively specific for hydrogen peroxide and reactive nitrogen species. Because reactive nitrogen species is minimally produced in hepatocytes, DCFH oxidation in HepG2 cells mainly detects hydrogen peroxide. In a subsequent experiment, we determined whether M1 or M2 abolished oxidative stress induced by AA. A flow cytometric assay using DCFH-DA indicated that pretreatment with either M1 or M2 effectively abrogated intracellular hydrogen peroxide production increased by AA treatment (12 h) (Fig. 2A). Interestingly, M2 treatment effectively decreased hydrogen peroxide production even as compared with
vehicle-treated control. As expected, oltipraz also inhibited DCFH oxidation induced by AA. Catalase treatment also completely inhibited DCFH oxidation induced by AA (Fig. 2B). To determine the antioxidative effects of M1 and M2, we next measured the level of GSH, an important endogenous antioxidant. AA-induced GSH depletion was antagonized by M1 or M2 (Fig. 2C). NAC, which was used as a positive control, also reversed the GSH depletion. Trolox, a vitamin E analogue and well-known antioxidant, exhibited a similar effect (Fig. 2D). Our data illustrating the decrease in ROS production by the metabolites of oltipraz demonstrate that the cytoprotective effects of M1 and M2 might result from their antioxidative capacity, by which cells maintain redox-homeostasis.

**Effects on AA-induced Mitochondrial ROS Production.** A previous study has shown that an intermediate metabolite of oltipraz, pyrrolopyrazine thione (PPD), interacts with cytochrome c (cyt c) by binding to and thereby reducing the heme iron (Velayutham et al., 2007). By this interaction, PPD inhibits the peroxidase activity of tight mitochondrial inner membrane-bound cyt c as well as the superoxide radical scavenging activity of oxidized cyt c. Consequently, PPD enhances ROS levels in mitochondria. A flow cytometric assay using MitoSOX (a live-cell-permeable and mitochondrial localizing superoxide indicator) showed that M1 or M2 pretreatment inhibited AA-induced mitochondrial ROS production (Fig. 3). In contrast, oltipraz showed no inhibitory effect. Rather, oltipraz treatment alone increased the mitochondrial MitoSOX fluorescence, which presumably results from the production of PPD (Velayutham et al., 2007). Either M1 or M2 treatment alone did not do so. Hence, there is a novel difference between oltipraz and its oxidized metabolites in terms of their mitochondrial ROS production. Mn(III) tetrakis (4-benzoic acid) porphyrin (Mn-TBAP; a novel superoxide dismutase mimetic) also protected cells against AA-induced mitochondrial ROS production (Fig. 3).

**Abrogation of AA-induced Mitochondrial Dysfunction by M1 or M2.** Previous studies have shown that AA treatment impairs mitochondrial respiratory activity, and thereby elicits mitochondrial dysfunction (Cocco et al., 1999). Rh123 as a membrane-permeable cationic fluorescent is used as a sensitive probe of mitochondrial membrane potential in populations of apoptotic cells because this agent binds to the mitochondrial membranes (Lemasters and Nieminen, 1997). Low staining intensity of Rh123 represents mitochondrial damage and dysfunction. In an attempt to correlate AA-induced
apoptosis with an alteration in mitochondrial function, MMP was measured using FACS after staining of the cells with Rh123. AA treatment (12 h) significantly increased the population of cells with relatively low Rh123 staining (left-gated) (Fig. 4A), which represents mitochondrial damage and dysfunction. Our results provided evidence that AA treatment induced mitochondrial dysfunction, which led to cell death. Further, we assessed whether each of the metabolites enables cells to restore MMP in response to a challenge by AA. M1 was effective in abolishing enhanced mitochondrial dysfunction induced by AA. Although M2 treatment at 30 μM slightly increased the population of Rh123-negative cells, M2 had a protective effect on the change in MMP induced by AA (Fig. 4B, left). The metabolites M3 and M4 had no effect on AA-induced mitochondrial dysfunction. Treatment with Trolox, which directly scavenges ROS, inhibited the ability of AA to increase Rh123-negative cells population, indicating that excess cellular ROS production may elicit mitochondrial impairment (Fig. 4B, right).

Cyclosporin A (CsA; an inhibitor of cyclophilin D that is a component of mitochondrial permeability transition pore) similarly inhibited AA-induced mitochondrial dysfunction (Fig. 4C). However, cyclosporin A treatment failed to inhibit DCFH oxidation increased by AA, suggesting that cellular ROS production elicited by AA may not result from mitochondrial dysfunction. Collectively, our results, corroborating the inhibitory effects of M1 and M2 on the change in MMP induced by AA, support the possibility that the cytoprotective effects of M1 and M2 result from the inhibition of cellular ROS production, which may lead to the restoration of mitochondrial function.

**AMPK Activation by M1 and M2 and the Role in AA-induced DCFH Oxidation.** Next, we assessed the effects of M1 and M2 on the time-responses of ACC and AMPK phosphorylations. M1 and M2 treatments increased the phosphorylation of ACC or AMPKα subunit, which represents the cellular AMPK activity (Fig. 5A). Apparently, the increases in ACC and AMPK phosphorylations were greater in cells treated with M2, as compared to those treated with M1. We also measured the phosphorylations after treatment with M1, M2, M3, M4 or oltipraz for 30 min. Both M1 and M2 effectively increased ACC and AMPK phosphorylations (Fig. 5A). Similarly, oltipraz exhibited an AMPK-activating effect. Neither M3 nor M4 was active.

To test the role of metabolite’s activation of AMPK in decreasing cellular ROS production, the
AMPK effect on DCFH oxidation was measured in cells treated with AA. The decrease in AA-induced DCFH oxidation by each of the metabolites was reversed by DN-AMPKα overexpression (Fig. 5B, upper). As expected, overexpression of DN-AMPKα blocked ACC phosphorylation increased by either M1 or M2 treatment (Fig. 5B, lower). Our results demonstrate that M1 and M2 metabolites have antioxidant effects, which may be associated with AMPK activation.

**The Role of AMPK Activation in AA-induced Mitochondrial Dysfunction.** To assess the role of the metabolites’ activation of AMPK in the restoration of mitochondrial function, we next determined the effect of compound C, an inhibitor of AMPK, on Rh123-negative cell populations. We found that M1 or M2 treatment decreased the count of Rh123-negative cell populations against the challenge of AA, and which was reversed by simultaneous treatment of compound C (Fig. 6A). The inhibitory effect of compound C for AMPK was confirmed by the decreased ACC phosphorylation (Fig. 6A, inset). By the same token, the inhibitory effects of M1 and M2 on Rh123-negative cell populations against AA were reversed by DN-AMPKα (Fig. 6B, left). In addition, we examined the effect of DN-AMPK on the ability of cyclosporin A to inhibit AA-induced mitochondrial dysfunction. Our result shows that DN-AMPK overexpression failed to reverse the beneficial effect of cyclosporin A (Fig. 6B, right), suggesting that AMPK is not directly involved in the events associated with the inhibition of mitochondrial permeability transition. Therefore, these data indicate that the activation of AMPK by M1 and M2 may contribute to the recovery of mitochondrial function against AA as a result of an increase in antioxidant capacity.

**Abrogation of AA-induced Apoptosis and DCFH Oxidation by AICAR.** In the subsequent experiment, we confirmed that AICAR, a well-known AMPK activator, had a similar cytoprotective effect against AA (Fig. 7A). AICAR treatment abolished DCFH oxidation, but failed to inhibit AA-induced mitochondrial superoxide production in cells (Fig. 7B, left). AICAR activation of AMPK was confirmed by ACC phosphorylation (Fig. 7B, right). Our findings provide further evidence that increased cell viability by AMPK activation against AA may result from the inhibition of cellular ROS production, but not that of mitochondrial superoxide production.
Discussion

Oltipraz has been studied as a chemopreventive agent for malignancies (Bolton et al., 1993; Kensler, 1997). Its cancer chemopreventive effect is considered to be mediated in part by the induction of phase 2 enzymes. In human studies conducted in Qidong, China, oltipraz treatment regimens with higher doses and a long-dosing interval were efficacious in preventing hepatocellular carcinoma (Jacobson et al., 1997), which is supported by the decreases in median levels of the phase 1 metabolite aflatoxin M$_1$ excreted in the urine of individuals receiving a higher dose of oltipraz (500 mg/week) (Wang et al., 1999). Because of lipophilicity, oltipraz tends to accumulate in several organs (Bae et al., 2004). Hence, treatment at higher concentrations may allow oltipraz to reside for a longer time in the body and result in the production of more of its metabolites. During metabolism, approximately 1% of oltipraz is converted to an oxo analog, M1, and the major metabolite is a dimethylated pyrrolopyrazine, M2, which includes a chemically rearranged form of the dithiolethione ring (O’Dwyer et al., 1997). Nevertheless, the possible cytoprotective effects of the oxidized metabolites of oltipraz have not been investigated yet.

AA initiates ROS generation and lipid peroxidation (Muralikrishna Adibhatla and Hatcher, 2006), which contributes to cell death (e.g., neurons) (Kwon et al., 2005). Activation of a non-selective cation conductance by AA results in cytoplasmic and mitochondrial overload of [Na$^+$] and [Ca$^{2+}$] (Fang et al., 2008). Apoptosis elicited by AA involves activation of caspase-3, which is amplified by release of mitochondrial cyt c and mitochondrial proteins (Scorrano et al., 2001). Hence, AA potently induces mitochondrial swelling and also has a direct effect on permeability transition pore opening in mitochondria (Scorrano et al., 2001). In addition, the loss of mitochondrial transmembrane potential caused by AA in cells may be associated with calcium accumulation in mitochondria and the consequent apoptosis (Maia et al., 2006). AA or its eicosanoid metabolite promotes inflammation or alters biological function in several organs (Brash, 2001). AA may also be directly cytotoxic. All of these results support the notion that excess AA may trigger the apoptotic pathway as a consequence of ROS production and mitochondrial dysfunction.

Recently, we found that oltipraz inhibits AA- or AA+iron-induced apoptosis (Shin and Kim, 2009). Here, we report that the oxidized metabolites of oltipraz exert cytoprotective effects against AA. Our
results shown here demonstrate that AA treatment increased cytotoxicity and apoptosis, as evidenced by PARP cleavage, caspase-3 activation and a decrease in the level of Bcl-xL with increases in DCFH oxidation and mitochondrial dysfunction. Interestingly, either M1 or M2 was capable of inhibiting apoptosis, cellular DCFH oxidation, and mitochondrial injury elicited by AA, despite the result that M2 treatment alone increased the populations of Rh123-negative cells. However, M3 and M4 were inactive, which was consistent with their inability to activate C/EBPβ or Nrf2. The major finding of this study is that, among the metabolites of oltipraz, M1 and M2 exert a cytoprotective effect against AA as a consequence of their antioxidative capacity.

Oltipraz has an antioxidant effect at least in part by inducing the phase 2 enzymes (Kang et al., 2003) and/or the manganese superoxide dismutase (Mn-SOD) gene (an antioxidant enzyme in the mitochondrial matrix that protects cells from oxidative damage)(Antras-Ferry J et al., 1997). Several lines of study have shown that the protective effects of oltipraz might be associated with its prooxidant activity and electrophilicity, which enhances phase 2 enzyme induction via Nrf2 activation and thereby elicits an antioxidant and anticarcinogenic effect (Velayutham et al., 2005a; Antras-Ferry J et al., 1997; Kim and Gates, 1997). Hence, the antioxidative capacity of oltipraz might require the oxidative metabolism of oltipraz, which is supported in part by the observations that oltipraz has a relatively short half-life (2.5 h) and very rapidly induces Mn-SOD (maximum after 4 h) (Antras-Ferry J et al., 1997). Our observations indicate that oltipraz, M1 or M2 all protect cells from AA-induced apoptosis. However, in contrast to the effect of oltipraz, M1 and M2 failed to protect cells against AA+iron, which may be due to their inability to scavenge highly reactive free radicals (e.g., hydroxyl free radical) produced by iron catalysis. This may account for the differential efficacy of oltipraz against AA+iron. The strong protective effect of oltipraz may be explained by its metabolism and adaptive enzyme induction. PPD, a metabolic intermediate of oltipraz’s conversion to M2, has the ability to induce the activity of phase 2 enzymes, react with GSH and thereby generate oxygen free radicals (Velayutham et al., 2005b). Recently, it was found that PPD inhibits the antioxidant activity of cyt c and thus enhances ROS levels in mitochondria (Velayutham et al., 2007). In the present study, we observed that oltipraz treatment promoted superoxide production in mitochondria (Fig. 3). Therefore, it is presumed that PPD induces phase 2 enzymes via mitochondrial ROS production and Nrf2 activation,
a key transcription factor for chemopreventive action (Fig. 8).

The AMPK system monitors cellular energy status by responding to changes in the AMP:ATP ratio (Shaw et al., 2004). The phosphorylation of AMPK regulates apoptosis in response to a lowered energy state by inhibiting anabolic pathways and stimulating catabolic processes (Shaw et al., 2004). AMPK activation may prevent apoptosis induced by glucocorticoid (Stefanelli C et al., 1998), hyperglycemia (Ido et al., 2002), and hepatic ischemia-reperfusion (Peralta C et al., 2001). In a previous study, oltipraz had a cytoprotective effect against AA and/or iron through AMPK activation (Shin and Kim, 2009). Another important finding of this study is that M1 and M2 activate AMPK and that the cytoprotective effect of the metabolites against AA also depends on AMPK. The essential role of AMPK in the cytoprotective effect was verified by the results obtained with a chemical inhibitor of AMPK as well as DN-AMPKα overexpression.

Neither M1 nor M2 increased mitochondrial superoxide generation, as shown in the MitoSOX assay (Fig. 3). Rather, these metabolites abrogated the ability of AA to induce mitochondrial superoxide production. AICAR treatment failed to inhibit AA-induced mitochondrial superoxide production (Fig. 7B), which supports the contention that the inhibition of AA-induced mitochondrial superoxide production by M1 and M2 may not depend on AMPK.

Our results illustrating the differential effects between oltipraz and its metabolites suggest that oltipraz, as the parent compound, may have another mechanism which enables cells to increase the adaptive response. The detailed mechanism remains to be investigated. In mitochondria, the electron transport chain complexes I and III are the main sites of superoxide radical production (Meany et al., 2006). AA also directly inhibits complex I and III in the respiratory chain of mitochondria (Cocco et al., 1999). In a previous study, oltipraz was found to attenuate apoptosis induced by rotenone (complex I inhibitor), but not that by antimycin A (complex III inhibitor) (Shin and Kim, 2009). In the present study, M1, but not M2, was able to attenuate apoptosis induced by rotenone (data not shown). Hence, it is possible that M1 also has a target in complex I or nearby site(s).

In conclusion, these results demonstrate that M1 and M2 exert cytoprotective effects and have the ability to increase antioxidant capacity and thereby recover mitochondrial function, which may be associated with AMPK activation (Fig. 8).
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Footnotes

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Legends for figures

Fig. 1. Inhibition of AA-induced cell death by oltipraz’s metabolites. A) The chemical structures of oltipraz and its major metabolites. B) Cell viability. Light microscopy shows the morphology of the cells (200×). HepG2 cells were treated with the metabolites (30 μM each) for 30 min, followed by the treatment of 10 μM AA for 24 h (upper). Cell viability was assessed using MTT assays. Cells were treated with the metabolites or 30 μM oltipraz for 30 min, followed by incubation with 10 μM AA for 24 h (lower). Data represent the mean ± S.E. of four separate experiments (significant compared to vehicle-treated control, **p<0.01; significant compared to the cells treated with AA, *p<0.05, ***p<0.01; no significant differences compared to the cells treated with AA, N.S.). C) TUNEL assays. Light microscopy shows TUNEL-positive cells (dark brown staining) (200×). HepG2 cells were treated as described in the legend to Fig. 1B (upper). The percentages of TUNEL-positive cells were quantified (lower). Data represent the mean ± S.E. of four separate experiments (significant compared to vehicle-treated control, **p<0.01; significant compared to the cells treated with AA, *p<0.05, ***p<0.01). D) Immunoblottings for proteins associated with apoptosis. Proteins were immunoblotted in lysates of HepG2 cells treated with 30 μM M1 or M2 for 30 min, followed by 10 μM AA treatment for 24 h. Results were confirmed by repeated experiments.

Fig. 2. Inhibition of AA-induced oxidative stress by M1 or M2. A) DCFH oxidation. DCFH oxidation was monitored in HepG2 cells treated with 30 μM M1, M2 or oltipraz for 30 min, followed by 10 μM AA treatment for 12 h. B) Inhibition of DCFH oxidation by catalase. HepG2 cells were incubated with PEG-catalase (1000 U/ml) for 30 min, followed by the addition of 10 μM AA for 12 h. C) GSH content. The GSH content was assessed in the cells that had been treated with 30 μM M1 or M2 for 30 min, followed by AA treatment for 24 h. Cells were also treated with 2 mM NAC in combination with metabolites. Data represent the mean ± S.E. of three separate experiments (significant compared to vehicle-treated control, *p<0.05, **p<0.01; significant compared to the cells treated with AA, *p<0.05, ***p<0.01). D) MTT assays. Cells were treated with 100 μM trolox for 30 min, followed by AA treatment for 24 h. Data represent the mean ± S.E. of four separate experiments (significant compared
to vehicle-treated control, **$p<0.01$; significant compared to the cells treated with AA, ##$p<0.01$).

**Fig. 3.** Inhibition of AA-induced mitochondrial ROS production by M1 or M2. HepG2 cells were treated with 30 μM M1, M2 or oltipraz for 30 min, followed by 10 μM AA treatment for 12 h. Cells were also treated with 20 μM Mn-TBAP with or without AA. The mitochondrial superoxide was assessed by using MitoSOX.

**Fig. 4.** Abrogation of AA-induced mitochondrial dysfunction by M1 or M2. A) Changes in mitochondrial membrane potential (MMP). HepG2 cells were treated with 30 μM M1, M2, M3 or M4 for 30 min, followed by 10 μM AA treatment for 12 h. After staining with Rh123 for 1 h, the cells were harvested. MMP was assessed by measuring the intensities of fluorescence from Rh123. Normal cells were located in the Rh123-positive field, whereas cells with mitochondrial damage were in the Rh123-negative field. B) The effects of oltipraz’s metabolites on Rh123-negative cell subpopulation. HepG2 cells were treated with the metabolites, oltipraz, or Trolox for 30 min, followed by AA treatment for 12 h. Data represent the mean ± S.E. of four separate experiments (significant compared to vehicle-treated control, **$p<0.01$; significant compared to the cells treated with AA, # $p<0.05$, ##$p<0.01$; no significant differences compared to the cells treated with AA, N.S). C) The effects of CsA treatment on Rh123-negative cell subpopulation and DCFH oxidation. The cells were treated with 2.5 μg/ml CsA for 30 min, followed by AA treatment for 12 h. Data represent the mean ± S.E. of four separate experiments (significant compared to vehicle-treated control, **$p<0.01$; significant compared to the cells treated with AA, ##$p<0.01$).

**Fig. 5.** AMPK activation and its effect on AA-induced DCFH oxidation. A) AMPK activation. Immunoblot analyses were performed in the lysates of cells that had been treated with 30 μM M1 or M2 for the indicated time periods (upper). Cells were treated with 30 μM M1, M2, M3, M4 or oltipraz for 30 min (middle). Relative phosphorylated AMPK band intensities of immunoblot data were quantified (lower) (significant compared to vehicle-treated control, **$p<0.01$; no significant
differences compared to vehicle-treated control, N.S). **B)** The effect of Ad-DN-AMPKα on DCFH oxidation. After Ad-LacZ or Ad-DN-AMPKα infection for 48 h, HepG2 cells were incubated with M1 or M2 for 30 min and thereafter continuously exposed to AA for 12 h. DN-AMPKα overexpression was confirmed by a decrease in the phosphorylation of ACC.

**Fig. 6.** The role of AMPK in the ability of M1 and M2 to protect mitochondria against AA. **A)** The effect of compound C (Cpd C) on Rh123-negative cell subpopulation. After 1 μM compound C treatment for 30 min, the cells were incubated with M1 or M2 for 30 min and thereafter continuously exposed to AA. Rh123-negative cell subpopulation was analyzed as described in the legend to Fig. 4A. AMPK inhibition was confirmed by a decreased ACC phosphorylation. Data represent the mean ± S.E. of four separate experiments (significant compared to vehicle-treated control, **p<0.01; significant compared to the cells treated with AA, *p<0.05, **p<0.01; significant compared to the cells treated with AA+M1 or AA+M2, *p<0.01). **B)** The effect of Ad-DN-AMPKα on Rh123-negative cell subpopulation. After Ad-LacZ or Ad-DN-AMPKα infection for 48 h, HepG2 cells were incubated with M1, M2, or CsA for 30 min and thereafter continuously exposed to AA for 12 h. Rh123-negative cell subpopulation was analyzed as described in the legend to Fig. 4A. HA immunoblotting verified DN-AMPKα overexpression (inset). Data represent the mean ± S.E. of four separate experiments (significant compared to respective vehicle-treated control, **p<0.01; significant compared to the cells treated with AA after Ad-LacZ infection, *p<0.05, **p<0.01; no significant differences compared to Ad-DN-AMPKα infection alone, N.S).
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AA for 12 h.

**Fig. 8.** The proposed scheme illustrating the mechanism by which M1 and M2 exert the cytoprotective effects. PPD, the intermediate metabolite, generates mitochondrial ROS production.
Fig. 1
Fig. 2
Fig. 4
Fig. 5
Fig. 6
Fig. 7