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Glutathione-Mediated Formation of Oxygen Free Radicals by the Major Metabolite of Oltipraz

Murugesan Velayutham,[†] Frederick A. Villamena,[†] Mettachit Navamal,[‡] James C. Fishbein,*[,‡] and Jay L. Zweier*[,†]

Center for Biomedical EPR Spectroscopy and Imaging, The Davis Heart and Lung Research Institute, and The Division of Cardiovascular Medicine, The Department of Internal Medicine, The Ohio State University College of Medicine, Columbus, Ohio 43210, and Department of Chemistry and Biochemistry, University of Maryland, Baltimore County, 1000 Hilltop Circle, Baltimore, Maryland 21250

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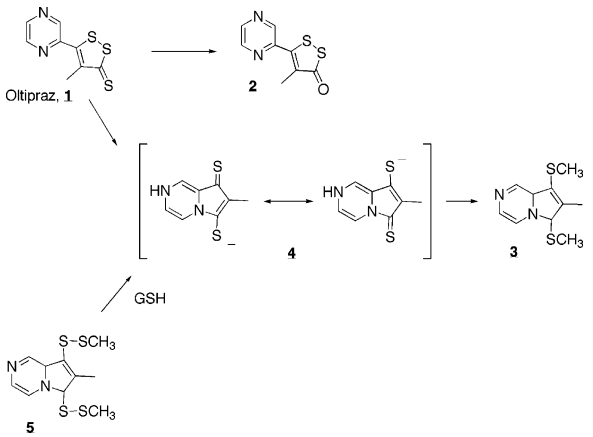
The major metabolite of the cancer chemopreventive oltipraz (**1**), a pyrrolopyrazine thione, **4**, has been shown to be a phase two enzyme inducer, an activity thought to be a key to the cancer chemopreventive action of the parent compound. To understand the possible mechanism by which the metabolite acts as an inducer, a study of its potential to generate free radicals was undertaken. Electron paramagnetic resonance (EPR) spin trapping studies using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) were performed with 7-methyl-6,8-bis-methyldisulfanyl-pyrrolo[1,2-*a*]pyrazine, **5**, a synthetic precursor to the metabolite in aqueous and organic solvents. In the presence of GSH, which rapidly liberates the metabolite from the precursor, a 1:2:2:1 quartet spectrum with hyperfine coupling constants $a_N = a_H = 14.9$ G, characteristic of the hydroxyl radical adduct of DMPO, was observed in the presence of oxygen. No signal was seen under anaerobic conditions. This signal was quenched by the addition of the superoxide scavenging enzyme Cu,Zn-superoxide dismutase. In aqueous dimethyl sulfoxide (80 vol % DMSO), the metabolite prescursor **5**, GSH, and DMPO exhibited an EPR spectrum with the hyperfine values of $a_N = 12.7$ G, $a_{H1} = 10.3$ G, and $a_{H2} = 1.3$ G, corresponding to the superoxide radical adduct of DMPO. The amount of superoxide radical adduct formed from the reaction of **5** and GSH increases with GSH concentration in phosphate buffer solution. Kinetic studies show that the formation of superoxide radical anion is first-order with respect to GSH. The formation of superoxide radical anion by the metabolite in the presence of GSH is linear at lower concentrations of **5** but becomes nonlinear at high concentrations. Overall, these studies suggest a mechanism in which GSH reduces the metabolite **4** to **4**$^{\bullet}$, presumably a radical anion, that in turn donates an electron to oxygen resulting in superoxide radical anion formation. This GSH stimulated redox cycle of the metabolite **4** suggests a possible mechanism by which the parent compound oltipraz might effect the cancer chemopreventve increase in the transcription of phase two enzymes that is mediated by transcription factor Nrf2.

Introduction

Many classes of cancer chemopreventive agents including phenolic antioxidants, 1,2-dithiole-3-thiones, isothiocyanates, flavones, and coumarins are effective at blocking DNA damage that initiates the carcinogenic process by modulating the metabolic activation and detoxication of carcinogenic substances (*1*−*3*). Oltipraz (**1**; Scheme 1) is a member of the class of compounds called dithiolethiones and is currently in phase two clinical trials in the People's Republic of China for the prevention of aflatoxin-induced hepatocellular carcinoma (*4*−*6*). Oltipraz also acts as a chemopreventive against colorectal cancer in a rat model (*7*−*9*).

Dithiolethiones are believed to afford protection from electrophilic and oxidative assault because they raise the levels of many phase two enzymes, the enzymes of xenobiotic metabolism that are traps of electrophiles and ROS and are also conjugating enzymes that prepare metabolites for export (*10*−*12*).

Scheme 1



The biochemical basis for cancer chemoprevention by dithiolethiones including oltipraz is becoming increas-

* To whom correspondence should be addressed. (J.L.Z.) Tel: 614-247-7857. Fax: 614-247-7845. E-mail: zweier-1@medctr.osu.edu. (J.C.F.) Tel: 410-455-2190. Fax: 410-455-2608. E-mail: jfishbei@umbc.edu.
[†] The Ohio State University College of Medicine.
[‡] University of Maryland, Baltimore County.

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ingly clear (*1*, *10*, *13*−*21*). The induction of phase two enzymes by dithiolethiones is mediated by a 41 base pair enhancer element known as the antioxidant response element (ARE) that is found upstream of the coding regions of many phase two genes. Activation mediated by the ARE is effected by transcription factor Nrf2. It has been demonstrated recently that Nrf2 deficient mice have an enhanced sensitivity to chemically induced carcinogenesis, reduced constitutive levels of phase two enzymes, and fail to exhibit appreciable induction of enzymes normally induced by dithiolethiones (*20*−*22*). Nrf2 is largely sequestered in the cytosol, bound to the chaperone Keap 1, a cysteine-rich protein, which is anchored to the cytoskeleton by binding to actin. Thiol reactive agents, including dithiolethiones, have been shown to untether Nrf2 and permit/induce its translocation to the nucleus (*20*, *23*).

The human metabolism of oltipraz was studied some time ago as it has been previously employed as an antischistosomal agent (*24*). During the metabolism of oltipraz, approximately 1% is converted to an oxo analogue (**2**; Sheme 1), which is itself a phase two enzyme inducer (*17*, *25*). The major isolated metabolite is a dimethylated pyrrolopyrazine (**3**; Scheme 1). It was recently shown to arise from biological methylation of the intermediate pyrrolopyrazine-thione (**4**; Scheme 1), an anion at physiological pH (conjugate acid pK_a = 4.32) (*26*). It has also recently been demonstrated that **4** is a phase two enzyme inducer with a potency on par with oltipraz itself (*26*, *27*). We are interested in the molecular details of the signaling process by which the intermediate **4** increases the levels of phase two enzymes.

Two general hypotheses have been advanced concerning the mechanisms of activation. The first notion suggests that oltipraz, or perhaps a product of its reaction with cellular thiols, acts as an electrophile, binding to a protein thiol, perhaps subsequently effecting the closure of a dithiol linkage (*15*, *28*, *29*). This concept was based on some circumstantial correlations and inference. The second suggestion was that oltipraz and other dithiolethiones induce transcription by initiating a flux of "reactive oxygen species" (ROS) (*30*). This was based on the observation that oltipraz, and other dithiolethiones, induce nicking of supercoiled DNA in a reaction that was dependent upon the presence of thiols, oxygen, and metal ions but which was inhibitable by catalase. Presumably the indicated peroxides could activate a redox sensitive transcription factor, for which there is precedent (*23*), or possibly alter the structure of thiol-rich Keap 1, thus effecting the release of Nrf2. Aside from these results and some earlier work by Fleury in alkaline ethanol (*31*−*33*), there has been relatively little investigation of the chemistry of dithiolethiones, which could clarify what might be relevant to the molecular mechanism of phase two enzyme induction.

The mechanism by which the pyrrolopyrazine-thione metabolite **4** might bring about Nrf2 nuclear translocation is also not presently clear. Compound **4** does not appear to be electrophilic in aqueous media in the presence of strong nucleophiles (*26*). We are thus interested in the possibility that **4** might produce ROS. Toward this end, we have employed the sensitive and specific technique of electron paramagnetic resonance (EPR) spin trapping. We report here that **4**, generated from the alternative precursor **5** (Scheme 1), reacts with GSH in the presence of oxygen, at physiological pH, to generate superoxide anion radical ($O_2^{\bullet-}$), a known biological messenger and precursor to hydrogen peroxide, another demonstrated biological effector.

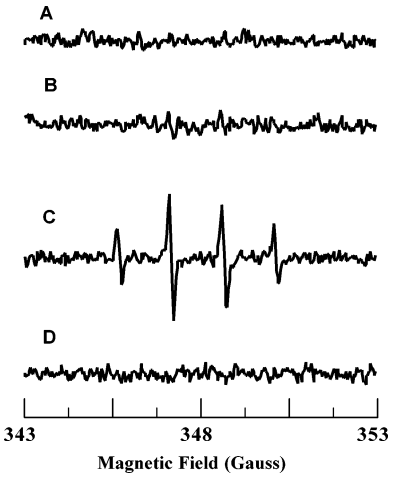


Figure 1. EPR spectra of DMPO adducts formed from **4**, generated from **5** and GSH in aqueous buffer. EPR measurements were carried out using a quartz flat cell at room temperature with **5** dissolved in aqueous 50 mM phosphate buffer with 10% acetonitrile. EPR instrument parameters used were as follows: microwave frequency, 9.775 GHz; modulation frequency, 100 kHz; modulation amplitude, 0.5 G; microwave power, 20 mW; number of scans, 10; scan time, 30 s; and time constant, 82 ms. EPR spectra were collected after 15 min. (A) Control, 40 mM DMPO, and 50 μM **5**; (B) control, 40 mM DMPO, and 0.1 mM GSH; (C) DMPO−OH adducts formation by 50 μM **5**, 0.1 mM GSH, and 40 mM DMPO; and (D) C in the presence of SOD1.

Experimental Procedures

Materials. All of the chemicals were obtained from commercial sources and were of analytical grade. Bovine erythrocyte copper,zinc-superoxide dismutate (SOD1) (98% enzyme, 4000−5800 units/mg), reduced glutathione (GSH), and oxidized glutathione (GSSG) were purchased from Sigma. Diethylenetriaminepentaacetic acid (DTPA) and 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) were obtained from Aldrich. Purified 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from Dojindo Laboratories (Kumamoto, Japan). Compound **5** was synthesized as described previously (*26*).

EPR Measurements. EPR spectra were recorded using quartz flat cells at room temperature with a Bruker ESP 300E spectrometer operating at X-band with 100 KHz modulation frequency and a TM_{110} cavity. Quantitation of the observed free radical signals was performed by computer simulation of the spectra and comparison of the double integral of the observed signal with that of a TEMPO standard (1 μM) measured under the identical conditions (*34*).

Results

The pyrrolopyrazine-thione metabolite **4** (Scheme 1) of oltipraz is formed instantaneously and quantitatively from the alternative precursor **5** (Scheme 1) at neutral pH in the presence of GSH (*26*). Subsequent EPR analysis of a solution containing 50 μM **5** and 40 mM DMPO to which had been added GSH to a final concentration of 100 μM showed a signal intensity ratio of 1:2:2:1 as shown in Figure 1C. No EPR signal was observed in the absence of **5** or GSH (Figure 1A,B). The addition of superoxide scavenging enzyme, SOD1, to the phosphate buffer solution (10% acetonitrile) containing 50 μM **5**, 0.1 mM GSH, and 40 mM DMPO, quenches the EPR signal (Figure 1D).

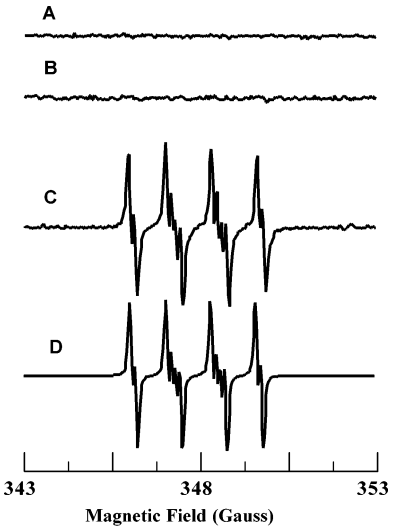


Figure 2. EPR spectra of superoxide radical adduct of DMPO formed from **4**, generated from **5** and GSH in DMSO. The medium is 80% DMSO, 10% acetonitrile, and 10% phosphate buffer. EPR spectra were collected after 15 min. EPR instrument parameters used were as described in the legend to Figure 1. (A) Control, 40 mM DMPO, and 50 μM **5**; (B) control, 40 mM DMPO, and 0.1 mM GSH; (C) DMPO−OOH adducts formation by 50 μM **5**, 0.1 mM GSH, and 40 mM DMPO; and (D) simulation of the EPR spectrum of the superoxide anion radical adduct of DMPO, DMPO−OOH, with parameters given in the text.

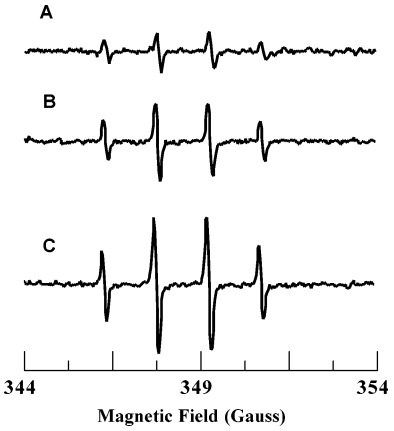


Figure 3. Effect of GSH concentrations on radical generation induced by **4**. EPR spectra of DMPO adducts were recorded in 10% acetonitrile−phosphate buffer for various concentrations of GSH with 40 mM DMPO and 50 μM **5**. (A) 1 mM, (B) 3 mM, and (C) 10 mM. EPR spectra were collected after 15 min. EPR instrument parameters used were as described in the legend to Figure 1.

The superoxide radical adduct of DMPO is more stable in organic-rich media than in aqueous medium (*35*). We therefore undertook experiments using organic solvents mixed with phosphate buffer in hopes of further establishing the type(s) of free radicals formed during the reaction between the metabolite **4** and GSH in phosphate buffer. The EPR spectrum of a solution containing 80% DMSO, 10% phosphate buffer, 10% acetonitrile, 50 μM **5**, 40 mM DMPO, and 0.1 mM GSH was recorded (see Figure 2C). The experimental spectrum was simulated theoretically as shown in Figure 2D using the EPR parameters $a_N = 12.7$ G, $a_{H1} = 10.3$ G, and $a_{H2} = 1.3$ G. No EPR signal was observed in the absence of **5** or GSH (Figure 2A,B).

Experiments were carried out to determine the order in **4** and GSH of the superoxide anion-generating reaction. EPR spectra of spin adducts of DMPO with various concentrations of GSH are shown in Figure 3. The plot of concentration of DMPO adducts vs time for various concentrations of GSH with 50 μM **5** and 40 mM DMPO in 10% acetonitrile−phosphate buffer solution is shown in Figure 4A. The plot of the initial rate of formation of DMPO−OH adducts vs various concentrations of GSH is shown in Figure 4B.

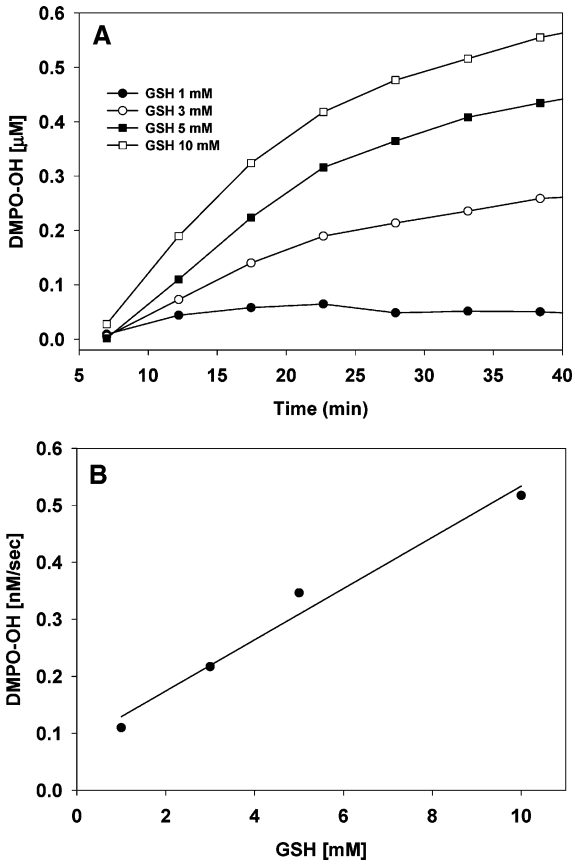


Figure 4. (A) Plot of the concentration of radical adducts vs time for various concentrations of GSH reacted with **5**. Experiments were performed with 50 μM **5**, 40 mM DMPO, and various concentrations of GSH in 10% acetonitrile−phosphate buffer solution. (B) Plot of the initial rate of formation of radical adducts vs GSH concentrations. EPR spectra were quantified by computer simulation of the spectra and comparison of the double integral of the observed signal with that of a TEMPO standard (1 μM) measured under the identical conditions. Rates in panel B were obtained from the initial slope of the data from panel A.

EPR spectra were also recorded for various concentrations of **5** at constant [GSH]. The plot of concentrations of DMPO−OH vs [**5**] at 5 mM GSH is shown in Figure 5A. The plot of the initial rate of formation of DMPO adducts vs various concentrations of **5** (up to 50 μM) with 40 mM DMPO and 5 mM GSH in 10% acetonitrile−phosphate buffer solution is shown in Figure 5B.

Discussion

In the experiments described in the present study, GSH is mixed with **5** at physiological pH, and it has been demonstrated that there is instantaneous conversion, in the millisecond time regime, to the pyrrolopyrazinethione, **4**, metabolite of oltipraz, which is relatively stable under these conditions (*26*). Thus, the present experiments detail the chemistry of the reaction of **4** with GSH in the time frame of minutes to hours, where it is feasible to employ standard EPR techniques.

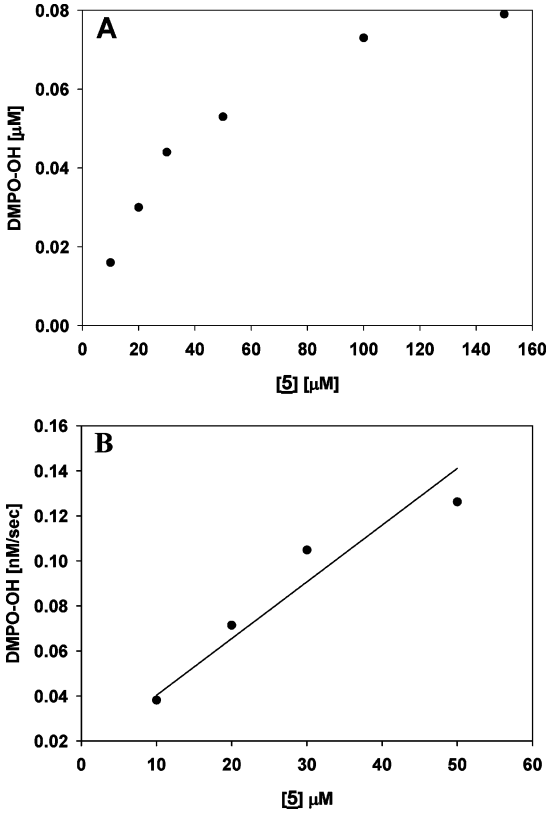
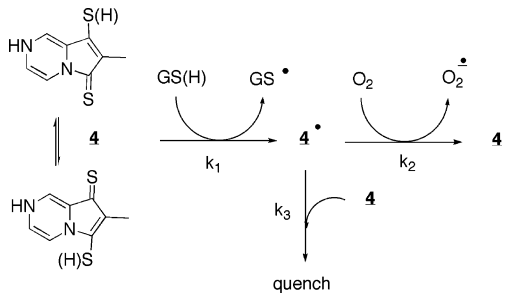


Figure 5. (A) Plot of the concentration of radical adducts vs concentrations of **4**, generated from **5**, with 5 mM GSH. Experiments were performed with 40 mM DMPO, 5 mM GSH, and various concentrations of **5** in 10% acetonitrile−phosphate buffer solution. (B) Plot of the initial rate of formation of radical adducts vs the initial concentration of **5** (up to 50 μM). EPR spectra were quantified by computer simulation of the spectra and comparison of the double integral of the observed signal with that of a TEMPO standard (1 μM) measured under the identical conditions. Rates in panel B were obtained from the initial slope of the data from panel A.

EPR spin trapping is a useful technique for the identification and characterization of short-lived ROS generated in solution. No EPR signal was observed for solutions in the presence of **5** or GSH alone, Figure 1A,B, or in the presence of all components in argon-flushed solutions (data not shown). The EPR spectrum in Figure 1C, in which **5** was incubated with excess GSH in buffered solutions containing ambient oxygen, shows a quartet signal with the intensity ratio of 1:2:2:1. The observed isotropic hyperfine values are $a_N = a_H = 14.9$ G, characteristic of the hydroxyl radical adduct of DMPO (DMPO−OH) (*36*). The formation of the DMPO−OH adduct in aqueous medium can occur by two pathways involving distinct ROS. First, the formed hydroxyl radical can be trapped directly by DMPO to yield DMPO−OH. Second, DMPO−OH can also form from spontaneous unimolecular decomposition of the superoxide radical anion adduct, DMPO−OOH, a reaction with a half-life of 45 s (*37*). The following experimental evidence demonstrates that the DMPO−OH is formed via the latter pathway.

(i) SOD1, which efficiently converts the superoxide radical anion into molecular oxygen and hydrogen peroxide (*38*), prevents the formation of DMPO−OH, as indicated in Figure 1D. (ii) Trapping of superoxide radical anion can be directly observed in more organic media, where the DMPO adduct is more stable. In DMSO, the superoxide radical anion adduct, DMPO−OOH, has been reported to have a half-life of 8 min (*35*). Figure 2 shows

Scheme 2



the EPR spectra obtained with 80 vol % DMSO, 10 vol % acetonitrile, and 10 vol % phosphate buffer. The isotropic EPR spectrum shown in Figure 2C consists of 12 lines. The isotropic hyperfine constants of the spectrum, $a_N = 12.7$ G, $a_{H1} = 10.3$ G, and $a_{H2} = 1.3$ G, are indicative of the superoxide radical anion adduct of DMPO, DMPO−OOH (*35*). The experimental spectrum was simulated based on the above parameters, as shown in Figure 2D, and gives excellent agreement with the experimentally observed spectrum. No EPR signal was observed when the solution was initially bubbled with argon (data not shown). This result further confirms that oxygen is essential for the formation of the superoxide radical anion.

Experiments summarized in Figures 3 and 4 indicate that the formation of superoxide radical anion is first-order in GSH. GSH stimulates the formation of superoxide radical as indicated by the spectra in Figure 3. EPR spectra in Figure 3 show that the intensity of the signal increases with an increasing concentration of GSH. The spectra shown in Figure 3 were taken from experiments that were carried out in 10% acetonitrile−phosphate buffer solution in the presence of **5**, DMPO, and GSH. The formation of DMPO−OH increases with time for various concentrations of GSH as shown in Figure 4A. The increase in signal intensity with the increasing of GSH shows that the free radical production is GSH dependent. A plot of the initial rate of formation of DMPO−OH as a function of GSH concentration is linear (Figure 4B), indicating the first-order dependence on GSH concentration.

The data summarized in Figure 5 indicate that there is a change in the dependence of the rate of $O_2^{\bullet-}$ formation upon the concentration of the pyrrolopyrazinethione (**4**). EPR spectra were recorded for various concentrations of **5** and with 5 mM GSH. The concentrations of free radical formation increase with an increasing concentration of **4**, formed from **5**, but reaches a plateau as shown in Figure 5A. A plot of the initial rate of formation of DMPO−OH as a function of the concentration of **4** (up to 50 μM) is approximately linear (Figure 5B), indicating a first-order dependence on the concentration of **4** at lower concentrations of **4**.

A mechanism consistent with the above observations is depicted in Scheme 2 in which the one electron reduction of oxygen by a reduced form of **4**, **4**$^{\bullet}$, generated from **4** and GS(H), competes with the quenching of **4**$^{\bullet}$ by **4**. The first-order dependence on [GSH], Figure 4B, is incorporated as the first step of the reaction that involves reduction of **4**, by what is presumably the thiolate form of GS(H). The requirement for GSH cannot be satisfied by alternative mechanisms in which GS(H) reduces an oxidized form of **4** (derived from the initial direct reduc-

tion of oxygen by **4**) because the rate of superoxide radical anion formation is sufficiently slow that the concentration of **4** is not limiting. The initial rates of formation of DMPO−OH are in tenths of μM/min, whereas the initial concentrations of **4** are hundreds of times greater. Under these conditions, the initial rate of formation of DMPO−OH would not be first-order in GSH. Another alternative can be ruled out. The initial reduction of oxygen by **4**, followed by slow GSH-mediated reduction of oxidized **4**, would be indicated by a burst of superoxide radical anion formation, and such a burst is not observed.

Scheme 2 posits the formation of a glutathiyl radical, and with the encouragement of a referee, additional experimental evidence consistent with its formation has been adduced. The spin trap DMPO can trap glutathiyl radicals to form the DMPO−GS adducts. However, the formation of the DMPO−GS adduct is highly reversible (*39*). In addition, while the DMPO−OH radical gives a 1:2:2:1 quartet, the DMPO−GS radical similarly yields a quartet but with a ratio of intensities of 1:1.4:1.4:1. Experiments carried out at higher **5**, DMPO, and GSH concentrations indeed give rise to a 1:1.6:1.6:1 quartet, consistent with a mixture of the two DMPO−radical adducts (see the Supporting Information). Additional indirect evidence of the glutathiyl radical arises from the formation of the formyl radical adduct of DMPO, DMPO−CO_2. The glutathiyl radical has been shown to abstract hydrogen from the formate ion to form the carbonyl radical that can be trapped by DMPO to form the DMPO−CO_2 radical adduct that gives a characteristic signal. EPR experiments carried out with solutions containing relatively high concentrations of **5**, GSH, and DMPO in the presence of 1 M sodium formate indeed yield spectra indicative of the formation of the DMPO−CO_2 radical adduct, and accompanying control experiments establish that hydroxyl radical is not the source of hydrogen abstraction, consistent with the glutathiyl radical as the initiating agent (see the Supporting Information).

The mechanism of Scheme 2 is also consistent with the change in dependence of the rate of formation of $O_2^{\bullet-}$ upon the concentration of **4** that is indicated by the data in Figure 5A. These data indicate that at low concentrations of **4** the velocity is first-order in the concentration of **4**, while at higher concentration the velocity becomes independent of the concentration of **4**. This behavior is consistent with the kinetic dominance, at higher concentrations of **4**, of a reaction in which a second molecule of **4** can quench the radical formed from the reduction by GS(H), as indicated by the lower path in Scheme 2. The k_3 process in Scheme 2, which is first-order in **4**, competes with O_2 for the radical **4**$^{\bullet}$ and, at sufficiently high concentrations of **4**, cancels the first-order dependence of superoxide radical anion production upon [**4**]. This can be seen in the rate law of eq 1 that is derived on the basis of Scheme 2, using the steady state assumption for **4**$^{\bullet}$. The increasing concentrations of **4** in the experiment summarized in Figure 5A are accompanied, of necessity, by increasing concentrations of disulfide, likely GSSG and $GSSCH_3$, due to removal of the thiomethyl groups of **5**, but control experiments indicate that increasing concentrations of GSSG from 5 to 25 mM in the presence of 5 mM GSH and 50 μM **5** have no detectable effect on the reaction velocity.

$$\frac{d[O_2^{-\bullet}]}{dt} = \frac{k_1 k_2 [O_2][\mathbf{4}][GSH]}{k_2[O_2] + k_3[\mathbf{4}]} \quad (1)$$

The results reported here show that in the presence of both GSH and the oltipraz metabolite **4**, superoxide radical anion is formed. This reaction could mediate the phase two enzyme inducing capacity of **4** that has been recently demonstrated (*27*). The chemoprotective induction of phase two enzymes via the transcription factor Nrf2 through the ARE can be initiated by other redox cycling agents, such as *tert*-butylhydroquinone (*23*, *40*−*42*), that are known to give rise to H_2O_2 by one electron reduction of oxygen and then dismutation of superoxide radical anion. Hydrogen peroxide readily modifies cysteine thiols and can accomplish the closure of a prospective dithiol sensor analogous to what appears to function in the prokaryotic Oxy R redox switch (*43*). Protein thiols of Keap 1 could be similarly involved in a redox (H_2O_2) sensitive switch that might thus be sensitive to the superoxide radical anion generating action of the metabolite **4** that has been established in the present work. Alternatively, it has recently been suggested that a similar process might intervene in the regulation of Nrf2 by protein kinase C, which is responsive to oxidative assault and which can initiate Nrf2 translocation via phosphorylation (*41*).

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Supporting Information Available: Experimental conditions, data, and analysis relevant to the formation of the glutathiyl radical in the reaction of **5** and GSH. This material is available free of charge via the Internet at http://pubs.acs.org.

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