Glutathione acts as a universal scavenger of free radicals at the expense of the formation of the glutathionyl radicals (GS\textsuperscript{•}). Here we demonstrated that GS\textsuperscript{•} radicals specifically interact with a reporter molecule, paramagnetic and non-fluorescent 4-((9-acridinecarbonyl)-amino)-2,2,6,6-tetramethylpiperidine-1-oxyl (Ac-Tempo), and convert it into a non-paramagnetic fluorescent product, identified as 4-((9-acridinecarbonyl)amino)-2,2,6,6-tetramethylpiperidine (Ac-piperidine). Horseradish peroxidase-, myeloperoxidase-, and cyclooxygenase-catalyzed oxidation of phenol in the presence of \( \text{H}_2\text{O}_2 \) and GSH caused the generation of phenoxy radicals and GS\textsuperscript{•} radicals, of which only the latter reacted with Ac-Tempo. Oxidation of several other phenolic compounds (e.g. etoposide and tyrosine) was accompanied by the formation of GS\textsuperscript{•} radicals along with a characteristic fluorescence response from Ac-Tempo. In myeloperoxidase-rich HL-60 cells treated with \( \text{H}_2\text{O}_2 \) and phenol, fluorescence microscopic imaging of Ac-Tempo revealed the production of GS\textsuperscript{•} radicals. A thiol-blocking reagent, \( \text{N} \)-ethylmaleimide, as well as myeloperoxidase inhibitors (succinyl acetone and azide), blocked formation of fluorescent acridine-piperidine. \( \text{H}_2\text{O}_2 \)/phenol-induced peroxidation of major classes of phospholipids in HL-60 cells was completely inhibited by Ac-Tempo, indicating that GS\textsuperscript{•} radicals were responsible for phospholipid peroxidation. Thus, GSH, commonly viewed as a universal free radical scavenger and major intracellular antioxidant, acts as a pro-oxidant during myeloperoxidase-catalyzed metabolism of phenol in HL-60 cells.

Thiols are said to fulfill important antioxidant functions in cells and biological fluids. In particular, glutathione, the most abundant intracellular low molecular weight thiol, regulates the content of \( \text{H}_2\text{O}_2 \) and organic (lipid) hydroperoxides via glutathione peroxidase-catalyzed reactions. GSH is also commonly presumed to act as a universal free radical sink toward a wide variety of different types of free radicals (1–5). Although scavenging of reactive radicals by GSH is believed to be an important antioxidant function of the latter, it may render effective protection only if the sulfur-centered glutathionyl radicals (GS\textsuperscript{•}) formed in the scavenging reactions do not further propagate free radical generation. On the contrary, pro-oxidant activity of GSH should be expected if GS\textsuperscript{•} radicals are reactive enough to interact with and cause damage to vital intracellular molecules.

In fact, experiments in model systems indicate that GS\textsuperscript{•} radicals can abstract hydrogen atoms from carbohydrates such as 2-deoxy-D-ribose and initiate irreversible DNA damage (6). Thiyl radicals are also capable of hydrogen abstraction from \( \text{a-amino-C-H} \) bonds of amino acids with the consequent formation of carbon-centered radicals (7). However, reactions of thyl radicals with carbohydrates and amino acids are reversible; moreover, carbon-centered radicals of carbohydrates and amino acids are stronger oxidants than thyl radicals, suggesting that thiols may “repair” carbohydrate- and amino acid- derived radicals.

It is likely that thyl radicals can effectively oxidize polyunsaturated fatty acids (PUFA) via abstraction of bis-allylic hydrogens, yielding pentadienyl radicals (8) with the subsequent formation of conjugated dienes, thiol-fatty acid adducts, and hydroperoxides (9, 10). In addition, thyl radicals can catalytically accelerate cis-trans isomerization of polyunsaturated fatty acid residues in phospholipids (9, 10). These pro-oxidant reactions of thyl radicals have been detected only in relatively simple chemical systems during the massive production of thyl radicals induced by pulse radiolysis or chemolysis with azo-initiators. Pro-oxidant activity of GS\textsuperscript{•} radicals has not been studied in detail under physiologically relevant conditions.

* This work was supported by National Institutes of Health Grants HL70755 and HL64145, as well as by the International Neurological Science Fellowship Program Grant F05 NS043922 administered by NINDS, National Institutes of Health in collaboration with the World Health Organization. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: GS\textsuperscript{•}, glutathionyl radical; Ac-Tempo, 4-((9-acridinecarbonyl)amino)-2,2,6,6-tetramethylpiperidine-1-oxyl; Ac-piperidine, 4-((9-acridinecarbonyl)amino)-2,2,6,6-tetramethylpiperidine; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; EPR, electron paramagnetic resonance; GS-NO, \( \text{S} \)-nitrosglutathione; HPLC, high pressure liquid chromatography; HRP, horseradish peroxidase; hSA, human serum albumin; MPO, myeloperoxidase; MS, mass spectrometry; mt, mitochondria; NEM, \( \text{N} \)-ethyl maleimide; PAPA NONOate, (Z)-1-[N-(3-ammonio propyl)]-N-(n-propyl)amidinodiazeyl-1-um-1,2-dioate; PBN, \( \text{a-phenyl-tert-butyl} \)-nitroso; PBS, phosphate-buffered saline; Ph, phenol; PMC, 3,2,5,7,8-pentamethyl-6-hydroxycromane; PnA, cis-parinaric acid; VP-16, etoposide or 4′-demethyllepidodophytoxin-9-(4,6-O-ethylidene-β-D-glucopyranoside).
The most commonly used approach to detecting GS radicals is based on their spin trapping (11–14). The low sensitivity of this technique and the instability of spin adducts are not sufficient to directly observe glutathionyl radicals in live cells (15). To overcome these technical difficulties, we chose to use a sensitive method for the monitoring of radicals by using fluoro-
phore-containing spin traps as probes to localize free radicals in cells (16, 17). We applied this approach to real time quanti-
tative monitoring of intracellular glutathionyl radicals by flu-
orescence microscopy based on the electron exchange effects in a reporter molecule, 4-(9-acridinecarboxyl)amino)-2,6,8-tet-
ramethylpiperidine-1-oxyl (Ac-Tempo), that specifically inter-
acts with GS radicals. Ac-Tempo contains two chemical moi-
eties conjugated by a peptide bond, namely fluorescent acridine and a stable paramagnetic nitroxide radical called Tempo, and, similar to SphinX, it inherits duality of their physical proper-
ties. The nitroxide radical, however, quenches the fluorescence of acridine through an intramolecular intersystem crossing induced by electron exchange (18). We established that the reaction of GS radicals with the nitroxide moiety of Ac-Tempo converts it to the corresponding piperidine, which therefore eliminates the quenching to yield a readily measurable fluores-
cence from acridine moiety and the disappearance of a typical EPR signal from the nitroxide radical. Thus, quantitative flu-
orescence microscopy (and fluorescence spectroscopy) of GS radical formation can be supported by EPR spectroscopy as well as by HPLC measurements.

Peroxidase-catalyzed, one-electron oxidation of phenolic compunds to the respective phenoxyl radicals has been re-
ported to initiate “futile thiol pumping” due to the interaction of phenoxyl radicals with GSH, yielding GS radicals (11, 19). To determine whether these reactions occur in live cells and to further study the interaction of GS radicals with intracellular constituents, we used Ac-Tempo as a reporter molecule specifically reacting with GS radicals upon the stimulation of my-
eloperoxidase (MPO) in HL-60 cells. We were able to visualize the formation of MPO-induced glutathionyl radicals during the H₂O₂-supported metabolism of phenol (Ph), and we established that GS radicals initiated the oxidation of phospholipids in live cells and caused cell death.

EXPERIMENTAL PROCEDURES
Reagents—Phenol, tyrosine, etoposide, hemin, sodium azide, magne-
sium chloride, glucose, sodium chloride, magnesium chloride, sodium hydride, potassium phosphate, HEPES, N-ethylmaleimide, acetoni-
trile (HPLC grade), diethylenetriaminepentaacetic acid, hydrogen per-
oxide, succinyl aceton, guaicol, glutathione, S-nitroso-glutathione, fatty acid-free human serum albumin (hSA), fetal bovine serum, 5,6-
dimethyl-1-pyrroline N-oxide (DMPO), a-phenyl-tert-butiyl nitronite (PBN), horseradish peroxidase (HRP) (type VI; EC 1.11.1.7), and my-
eloperoxidase (from human leukocytes, EC 1.11.1.7) were purchased from the Cayman Chemical Company. Polyoxyethylene (PBN), horseradish peroxidase (HRP) (type VI; EC 1.11.1.7), and my-
eloperoxidase (MPO) in HL-60 cells. We were able to visualize further study the interaction of GS radicals upon the stimulation of my-
eloperoxidase (MPO) in HL-60 cells. We were able to visualize

Fluorescence Microscopy—Cells were pre-incubated with Ac-Tempo (100 μM) in PBS for 5 min, and then Ph (100 μM) and H₂O₂ (20 μM) were added and incubated for 5 min. After treatments, cells were washed and resuspended in PBS for microscopy assay. Fluorescence was evaluated using a Nikon ECLIPSE TE 200 fluorescence microscope (Tokyo, Japan) equipped with a digital Hamamatsu CCD camera (C4742-95-
12NRB). Data were analyzed using Metamaging Series™ software

version 4.6 (Universal Imaging Corp.). At least 200 cells per experimen-
tal condition were counted.

Fluorescence Spectroscopy—A Shimadzu spectrofluorimeter RF-
5301PC was employed for fluorescence measurements in a 0.2-ml quartz cuvette. Ac-Tempo fluorescence spectra were detected using an excitation wavelength of 361 nm (excitation slit of 1.5 nm and an emission slit of 3 nm). The time course of fluorescence intensity was monitored using an emission wavelength of 440 nm. Concentration of Ac-Tempo was determined by measurements of optical density at 359 nm (ε = 10.4 nmol·cm⁻¹) using a Shimadzu UV160U spectrophotometer.

To determine concentrations of Ac-Tempo and 4-(9-acridinecarbon-
yl)amino)-2,6,8-tetramethylpiperidine (Ac-piperidine) in cells, we determined fluorescence spectroscopy of the latter corrected for the contrib-
uting fluorescence of Ac-Tempo. This value was estimated based on ex-
perimentally determined intracellular concentrations using the charac-
teristic EPR signals of Ac-Tempo (see below).

EPR Spectroscopy—EPR measurements were performed on a JEOL-
REX spectrometer (Kyoto, Japan) at 25 °C in glass capillary tubes (1.2-mm internal diameter; Fischer Scientific Co.). The tube (~ 12 cm in length) was filled with 50 μl of mixed sample and placed in an opened and sealed quartz cuvette. EPR spectra of Ac-Tempo, as well as spin traps, were recorded under the following conditions: center field, 335.5 mT; sweep width, 8 mT; sweep width; field modulation, 0.2 mT; microwave power, 10 milliwatts; time constant, 0.1 s; and time sweep, 1 min.

HPLC Analysis of Ac-Tempo Derivatives—HPLC was performed on a Shimadzu liquid chromatograph LC-600 equipped with absorbance di-
ode array detector SPD-M10A (Tokyo, Japan). The sample was applied to a reverse phase Alltech Econosphere C18, 5 μm column (5-μm; 4.6 × 250 mm) equilibrated with a solvent A (75 mM ammonium acetate, 30 mM citric acid, and 5% acetonitrile, pH 3.5). The column was eluted during the first 5 min with solvent A and then from min 5 to min 20 with a linear gradient from 0 to 35% of solvent B (100% acetonitrile). Flow rate was 1 ml/min. Injection volume was 20 μl. The elution profile was monitored by optical density in the range 200–400 nm. To prepare cell extracts for the HPLC assay, HL-60 cells (10⁶ cells) were centrifuged at 1,500 × g for 5 min, resuspended in PBS by ultrasonication, and centrifuged at 10,000 × g for 5 min. The superna-
tant obtained was treated with acetonitrile (1:1) for 5 min and cen-
trifuged at 10,000 × g for 5 min. Thus obtained supernatant was evapor-
ated under nitrogen gas and resuspended in 30 μl of PBS for the injection.

Measurements of Phospholipid Peroxidation Using Metabolic Labeling with PnA—PnA was incorporated into phospholipids of HL-60 cells by the addition of its human hSA complex as described previously (21). Briefly, cells in log phase of growth were rinsed twice with serum-free RPMI 1640 medium without phenol red and resuspended in this me-
dium to give a cell density of 10⁶ cells/ml. PnA-hSA complex was added to the cell suspension to give the final concentration of PnA per millilitre, and the cells were incubated at 37 °C in the dark under aerobic conditions. At the end of incubation period, the cells were conseqently washed twice with PBS containing fatty acid-free hSA (0.5 mg/ml) and not containing hSA, respectively, and resuspended in PBS containing 50 μM diethylenetriaminepentaacetic acid. Then cells were incubated with H₂O₂ and Ac-Tempo (100 μM) for 2 h at 37 °C. H₂O₂ (25 μM) was added every 30 min. At the end of incubation, lipids were extracted from cells using a slightly modified Folch procedure. Extracted lipids were dissolved in 0.2 ml of 2-propanol/hexane/water (4:3:0.6; v/v). Lipid phosphorus was determined using a previously described method (22) with slight modifications. Lipid extracts were separated by HPLC using an analytical column essentially as described previously (23). The lipid extract was applied to a 5-mm Supelcosil LC-Si column (4.6 × 3 × 250 mm) equilibrated with a mixture of 1 part of solvent A (2-propanol/hexane/water) (57:43:1, v/v) and 9 parts of solvent B (2-propanol/hexane/40 mM aqueous ammonium acetate, pH 6.8) (57:43:10, v/v). The column was eluted during the first 5 min with solvent A and then from min 5 to min 20 with a linear gradient from 10 to 37% solvent B; the period from min 3 to min 15 was isocratic at 37% solvent B; from min 15 to min 23 the linear gradient rose to 100% B; and then the period from min 23 to min 45 was isocratic at 100% solvent B. The solvent flow rate was 1 ml/min. A Shimadzu high-performance liquid chromatograph (LC-650) equipped with fluorescence detector (RF-551) was used. The fluorescent compound was monitored at excitation of 420 nm after excitation at 324 nm.

Mass Spectral Analysis—Ac-Tempo and derivatives were analyzed by electro spray ionization mass spectrometry by direct infusion into a Quattro II triple quadrupole mass spectrometer (Micromass, Inc.,
incubated in the dark (a) or in the presence of 100 mM DMPO (b) and time scan, 1 min. Lines representing DMPO-GS adduct in the following conditions: center field, 335.5 mT; sweep width, 8 mT; field modulation, 0.2 mT; microwave power, 10 milliwatts; time constant, 0.1 s; and time scan, 1 min. Lines representing DMPO-GS adduct on the EPR spectrum are marked with *a*, *b*, fluorescence spectra of 10 μM Ac-Tempo solutions, irradiated in the presence of 0, 10, and 20 μM GS-NO (a, b, and c, respectively). C, fluorescence spectra of 10 μM Ac-Tempo solutions incubated without (a) or with 0.2 mM PAPANONate (b); Ac-Tempo and 10 μM GS-NO were irradiated in the absence (c) and presence of 100 mM DMPO (d). a.u., arbitrary units.

Manchester, England). The electrospray probe was operated at a voltage differential of 3.5 keV (positive ion mode). Mass spectra were obtained by scanning the range of 150–850 m/z every 2.6 s and summing individual spectra. Source temperature was maintained at 70 °C. Collision-induced dissociation spectra were obtained by selecting the ion of interest and performing daughter ion scanning in Q3 using argon gas in the collision chamber. The spectrometer was operated at unit mass resolution in Q1 and slightly below unit resolution in Q3. To prepare cell extracts for MS analysis, HL-60 cells (3 × 10⁶ cells) were homogenized in 10 mM Tris-HCl (pH 7.4), centrifuged at 1,000 g for 5 min, resuspended in water, and extracted using a Folch procedure. Methanol and then chloroform were added to a cell suspension (1:2:1) and mixed; the mixture was incubated for 15 min under N₂. After centrifugation for 5 min at 1,500 × g, the lower chloroform layer was collected, and the extraction was repeated with the upper aqueous methanol layer. Finally, the combined chloroform layers were evaporated under a stream of N₂ and resuspended in 30 μl of acetonitrile.

**Statistics**—Data are expressed as means ± S.E. Changes in variables for different assays were analyzed either by Student’s t test (single comparisons) or one-way analysis of variance (ANOVA) for multiple comparisons. Differences among means were considered to be significant at p < 0.05.

**RESULTS**

**Biochemical Evidence for Selective Interactions of Ac-Tempo with GS Radicals during Peroxidase-catalyzed Oxidation of Phenol in the Presence of H₂O₂ and GSH**—The conjugate of Ac-Tempo and acridine, Ac-Tempo, is a paramagnetic compound that has a characteristic triplet EPR signal originating from its nitroxide moiety with the hyperfine constant a_V^A_{Ac-Tempo} = 16.9 G (Fig. 1A). Ac-Tempo does not exert fluorescence from the acridine moiety (Fig. 1B) due to the quenching produced by electron exchange interactions between the tempo radical and the excited state of acridine (18). The fluorescence quenching of Ac-Tempo can be eliminated upon conversion of the nitroxide moiety into a non-paramagnetic species, e.g., in the course of its interaction with another radical.

**GS Radicals Interact with Ac-Tempo**—Because of our interest in the reactions of glutathionyl radicals, we determined whether the radicals could convert Ac-Tempo into a non-paramagnetic fluorescent product. To this end, we generated glutathionyl radicals using UV-induced homolytic decomposition of S-nitrosylated glutathione (GS-NO). Irradiation of GS-NO in the presence of Ac-Tempo eliminated the EPR signal from the nitroxide moiety and induced characteristic fluorescence of the acridine moiety (Fig. 1A and B). The effect was dependent on the amount of GS-NO added (Fig. 1B). Furthermore, the addition of an excess of spin trap, DMPO, which forms a stable adduct with glutathionyl radicals (DMPO-GS-NO), yielded an EPR signal characteristic of the DMPO-GS-NO adduct with the splitting constants a_N = 15.4 G and a_H = 16.2 G (Fig. 1A, marked with +). Notably, DMPO inhibited the conversion of Ac-Tempo into a non-paramagnetic fluorescent product (Fig. 1A and C). In control experiments, UV-irradiation of Ac-Tempo alone or the incubation of Ac-Tempo with an NO donor, PAPANONate, did not trigger fluorescence response (Fig. 1C); neither did these treatments affect the Ac-Tempo EPR spectra (not shown). These results demonstrate that GS radicals were able to directly react with Ac-Tempo.

**GSH Is Essential for the Conversion of Ac-Tempo into a Non-paramagnetic Fluorescent Product during HRP-catalyzed Oxidation of Phenol**—We further determined to what extent GS radicals generated by a biologically more relevant oxidation system, i.e., peroxidase-catalyzed, one-electron oxidation of phenol to its phenoxyl radical in the presence of H₂O₂ and GSH, can convert Ac-Tempo into a non-paramagnetic fluorescent product (Scheme 1). The incubation of Ac-Tempo with HRP, H₂O₂, phenol, and glutathione induced decay of the EPR signal from Ac-Tempo and the emission of its fluorescence (Fig. 2, A and B). Peroxidase activity was essential, because the effect was not observed in the absence of peroxidase or H₂O₂. Fluorescence emission was not induced by HRP and H₂O₂ without phenol and GSH, suggesting that Ac-Tempo is not a “good” substrate for peroxidase; it was also not induced by HRP and H₂O₂ and phenol, indicating that the interactions of Ac-Tempo with phenoxyl radicals did not cause fluorescence response. GSH alone did not cause a reduction of Ac-Tempo that was observable by fluorescence and EPR spectroscopy even at concentrations as high as 5 mM (data not shown). Thus, the presence of GSH in the incubation system containing HRP, H₂O₂, and phenol was critical to the Ac-Tempo modification. Moreover, EPR signal decay and the magnitude of fluorescence emitted depended strongly on the amount of GSH added (Fig. 2, C and D).

**Ac-Tempo Interacts with GS Radicals Generated by Peroxidase-catalyzed Oxidation of Phenol**—Peroxidases catalyze the oxidation of phenol to a phenoxyl radical, which can be recycled back to phenol by GSH with the concomitant formation of a
Converting it into a fluorescent Ac-piperidine. GSH oxidation products (GS-X) are also formed during this reaction. In addition, GSH/H2O2/H2O2/H2O2/H2O2 radicals interact with non-fluorescent Ac-Tempo, generating superoxide anion radical (O2•−) upon reaction with O2.

Glutathionyl Radical-induced Oxidative Stress

**Scheme 1.** Depicted is the mechanism of Ac-Tempo interaction with peroxidase-induced glutathionyl radicals. Peroxidases catalytically reduce H2O2 to H2O. During this reaction, the catalytic site of the enzyme is converted into highly reactive oxidants, compound I and compound II, that are recycled back to the resting state at the expense of the oxidation of phenol (Ph-OH) to phenoxyl radicals (Ph-O•). Ph-O• radicals are reduced back to phenol by GSH with the concomitant formation of glutathionyl radicals (GS•). GS• radicals interact with non-fluorescent Ac-Tempo, converting it into a fluorescent Ac-piperidine. GSH oxidation products (GS-X) are also formed during this reaction. In addition, GS• radicals can recombine to yield GSSG disulfide or react with GSH, yielding a glutathione disulfide anion radical (GSSG•−), a reducing radical capable of generating superoxide anion radical (O2•−) upon reaction with O2.

Fig. 2. The effect of the HRP-catalyzed oxidation of phenol in the presence of H2O2 and GSH on the EPR spectra and the fluorescence emission of Ac-Tempo is dependent on GSH concentration. A, EPR signals. B, fluorescence spectra. The reaction mixture contained 10 μM Ac-Tempo pre-incubated without (a) or with 0.1 units/ml HRP, 50 μM phenol, 10 μM GSH, and 10 μM H2O2 for 10 min (b). C and D, time course of Ac-Tempo EPR signal decay and fluorescence emission produced by HRP/phenol/H2O2 in the presence of a range of GSH concentrations (a, 0 μM; b, 1.25 μM; c, 2.5 μM; d, 5.0 μM; and e, 10.0 μM). Other conditions were 0.1 units/ml HRP, 10 μM Ac-Tempo, 50 μM phenol, and 10 μM H2O2. Fluorescence was detected using an excitation wavelength of 361 nm and an emission wavelength of 440 nm. Conditions for EPR spectroscopy are the same as described in the Fig. 1 legend. a.u., arbitrary units.

Glutathionyl radical (11, 13, 15, 19) (Scheme 1). If peroxidase-generated GS• radicals are reactive toward Ac-Tempo, then their scavenging should inhibit conversion of Ac-Tempo into a fluorescent product. Indeed, large excess of DMPO and PBN, spin trapping agents reactive toward GS• radicals, significantly inhibited peroxidase-induced fluorescence response (Fig 3) and resulted in the accumulation of characteristic DMPO and PBN spin adducts with GS•. It is important to note that when phenol was omitted from the incubation system, HRP, H2O2/H2O2/H2O2/H2O2 and GSH did not induce a fluorescence response from Ac-Tempo. This is in agreement with earlier reported data, which indicated that GSH is a relatively poor substrate for HRP (13, 15, 24). Overall, these results demonstrate that GS• radicals play an important role in the conversion of Ac-Tempo into a fluorescent product.

**Fig. 3.** Effect of the spin traps DMPO and PBN on the fluorescence emission from products formed by the HRP-catalyzed metabolism of Ac-Tempo in the presence of H2O2/phenol and GSH. a, time course of Ac-Tempo fluorescence was recorded in the presence of HRP/phenol/H2O2; b, same as a plus GSH; c, same as a plus DMPO; d, same as a plus GSH and DMPO; e, same as a plus GSH and PBN; f, same as a plus DMPO; g, same as a plus PBN. Incubation conditions were 0.2 units/ml HRP, 10 μM Ac-Tempo, 50 μM phenol, 10 μM GSH, 10 μM H2O2, 100 mM PBN, and 100 mM DMPO. a.u., arbitrary units.

**Glutathione Disulfide Anion Radical and Superoxide Anion Radical Are Not Involved in the Conversion of Ac-Tempo into a Non-paramagnetic Fluorescent Product—**Because the reaction of GS• radicals with GSH yields the glutathione disulfide anion radical (GSSG•−), a reducing radical capable of generating a superoxide anion radical (O2•−) in the presence of molecular oxygen (Scheme 1), we tested further to determine whether these two radicals could interact with Ac-Tempo during the peroxidase-catalyzed oxidation of phenol. To this end, we increased GSH concentration significantly to favor GSSG formation (a predominant reaction of GS• radicals at high GSH levels; Refs. 1 and 2) and to facilitate its interaction with Ac-Tempo. The higher the concentration of GSH used, the lower the rate of fluorescence production observed (Table I). This suggests that, at high concentrations, GSH was able to out-compete Ac-Tempo in the reaction with GS•. An alternative interpretation, however, is that the rate of GS• radical generation increased at higher GSH concentrations resulting in GS• radical recombination and dimerization to GSSG, a competitive reaction with Ac-Tempo. Uncertainty of this result with respect to the role of GSSG was eliminated by an analysis of Ac-Tempo modification products using MS and HPLC. Only 10% of the products formed were represented by Ac-Tempo hydroxylamine, an an-

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TABLE I  
Effect of GSH on HRP-catalyzed metabolism of Ac-Tempo as evidenced by a fluorescence emission assay

| GSH  | Ac-Tempo modification, fluorescence assay \( \text{nmol/min} \) |
|------|---------------------------------------------------------------|
| \( \mu \text{M} \) | \( \text{nmol/min} \) |
| 10   | 3.60 ± 0.04                                                  |
| 100  | 1.84 ± 0.16                                                  |
| 1000 | 0.11 ± 0.04                                                  |

* Effect of high GSH concentrations on the rate of fluorescence emissions in the system of 10 \( \mu \text{M} \) Ac-Tempo, 0.2 units/ml HRP, 50 \( \mu \text{M} \) phenol, and 10 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \).

TABLE II  
Effect of superoxide dismutase on HRP-catalyzed metabolism of Ac-Tempo as evidenced by EPR signal decay and fluorescence emission assays

| Superoxide dismutase | Ac-Tempo modification \( \text{nmol/min} \) |
|----------------------|-------------------------------------------|
| units/ml             | EPR assay                                | Fluorescence assay |
| 0                    | 3.23 ± 0.42                              | 3.88 ± 0.46        |
| 10                   | 2.69 ± 0.50                              | 3.37 ± 0.36        |
| 100                  | 3.00 ± 0.74                              | 3.91 ± 1.02        |

* Effect of superoxide dismutase on the rate of Ac-Tempo metabolism in the same system as in Table 1 in the presence of 10 \( \mu \text{M} \) GSH.

Ac-TEMPO was found to be a potent radical scavenger with a high reactivity toward thiols such as GSH and cysteine, but not protein thiols. We further tested, by EPR spectroscopy, whether the paramagnetic signal of the nitroxide moiety of Ac-TEMPO was depleted accordingly. Indeed, a decrease in the EPR signal intensity of nitroxide was detected as a result of peroxidase stimulation in cells treated with phenol/\( \text{H}_2\text{O}_2 \). The magnitude of the EPR signal inversely correlated with the fluorescence emission and was affected by pre-treatment with azide or NEM, similarly as in the above experiments (Fig. 7B).

Specificity of the interaction between Ac-TEMPO and thyl radicals of GSH in cells was confirmed by MS and HPLC assay. MS analysis of the samples extracted by chloroform/methanol (2:1) from HL-60 cells after treatment with \( \text{H}_2\text{O}_2 \) and phenol revealed only one major derivative of Ac-TEMPO, which was represented by two species at \( \text{m/z} \) 181 and \( \text{m/z} \) 362, corresponding to double and single charged Ac-piperidine, respectively (Fig. 4, B and C). This was confirmed by tandem MS spectrometry. Interestingly, after incubation, the relative intensities of peaks corresponding to Ac-piperidine were higher than that of the peak of Ac-TEMPO (\( \text{m/z} \) 377) by almost an order of magnitude, indicating that Ac-TEMPO was converted into Ac-piperidine. In line with this finding, an analysis of HL-60 cell extracts by HPLC assay showed that the major fluorescent product of Ac-TEMPO metabolism (75%) was Ac-piperidine (Fig. 5, d and e). Only negligible amounts of Ac-piperidine were...
Fig. 4. Mass spectra of Ac-Tempo and its products formed by horseradish peroxidase-catalyzed oxidation in a model system (A) or by MPO-catalyzed metabolism in HL-60 cells (after extraction from HL-60 cells) (B and C). A, mass spectrum of the incubation system containing 100 μM Ac-Tempo, 200 μM GSH, 0.2 units/ml HRP, 50 μM phenol, and 10 μM H₂O₂ (after 30 min of incubation); B, mass spectrum of the extract from HL-60 cells treated with 20 μM Ac-Tempo (15 min of incubation). C, same as B, but the cells were treated with 100 μM phenol and 200 μM H₂O₂ in addition to Ac-Tempo (15 min of incubation). The peak at m/z 377 represents Ac-Tempo; peaks at m/z 181 and m/z 362 represent doubly and singly charged Ac-piperidine, respectively; the peak at m/z 378 represents the hydroxylamine of Ac-Tempo.
In separate experiments, the intracellular contents of Ac-Tempo were quantitatively estimated by simultaneous EPR spectroscopy and fluorescence spectroscopy. Incubation conditions for fluorescence microscopy were as follows: a, 100 μM Ac-Tempo; b, same as a plus 100 μM phenol and 20 μM H2O2; c, same as b plus pre-treatment with 50 μM NEM; d, same as b plus pre-treatment with 0.4 mM succinyl acetone for 48 h; e, same as a plus phenol; and f, phenol and H2O2 without Ac-Tempo. Incubation time with reagents was 5 min. Incubation conditions for EPR spectroscopy and fluorescence spectroscopy were the same as described above, except that the Ac-Tempo concentration was 10 and 20 μM for EPR and fluorescence measurements respectively; the azide concentration was 2 mm. **, p < 0.05 versus cells treated with H2O2. ***, p < 0.05 versus cells treated with PhH2O2.

Intracellular fluorescence response was dependent on the concentration of H2O2 added (Fig. 8B). H2O2 also governed oxidation of GSH, as was determined by a fluorescent Thio-Glo™-1 assay. Most importantly, Ac-Tempo partially protected GSH from oxidation, whereby the amount of GSH consumed was nearly equal to the amount of Ac-piperidine produced (Fig. 8, B and C).

Overall, these data suggest that low molecular weight thiols were critically involved in the reaction of Ac-Tempo, whereas phenoxyl radicals generated by the myeloperoxidase-catalyzed oxidation of phenol do not react directly with Ac-Tempo in HL-60 cells. Because GSH is the major target for phenoxyl radicals in cells resulting in the production of GS\(^{–}\) (13), the radicals of GSH are likely to react with Ac-Tempo, similarly as in the model system with purified peroxidase.

**Ac-Tempo Blocks Phospholipid Peroxidation and Protects HL-60 Cells against H2O2/Phenol-induced Cytotoxicity**—Assuming that Ac-Tempo reacts specifically with glutathionyl radicals in peroxidase-catalyzed reactions in HL-60 cells, we chose to use it as a trap to evaluate the potential role of GS\(^{–}\) radicals in phospholipid peroxidation. We metabolically labeled phospholipids in HL-60 cells with the oxidation-sensitive fluorescent fatty acid PnA and exposed them to phenol/H2O2. The loss of fluorescently labeled major classes of phospholipids upon oxidation was assayed by fluorescent HPLC (Fig. 9). A combination of H2O2 and phenol induced a dramatic (2–4-fold) oxidation of all major phospholipid classes, i.e. phosphatidyl-

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**Fig. 5.** HPLC profiles of Ac-Tempo and products of its peroxidase-catalyzed metabolism in model systems and in HL-60 cells. a, 30 μM Ac-Tempo (1). b, Ac-Tempo-H2O2 (2) obtained by the reduction of 130 μM Ac-Tempo with 650 μM ascorbate. c, Ac-piperidine (3) produced from 130 μM Ac-Tempo by HRP-catalyzed reaction in the presence of phenol, H2O2, and GSH. Incubation conditions were 130 μM Ac-Tempo, 0.3 units/ml HRP, 50 μM phenol, 130 μM H2O2, and 200 μM GSH. Incubation time was 15 min. d and e, HPLC profiles of extracts obtained from HL-60 cells (2 × 10^6 cells/ml) incubated with 100 μM Ac-Tempo and 20 μM H2O2 with (d) or without (e) 20 μM H2O2. Peaks are marked similar to standards presented on HPLC runs a–c. Note that Ac-Tempo was not observed on the chromatographs because it was washed away before the extraction procedure.

**Fig. 6.** Fluorescence responses produced in the course of interaction of Ac-Tempo with GS\(^{–}\) radicals in the presence of different peroxidases and their substrates. A, fluorescence responses induced by HRP (0.1 units/ml) in the presence of different phenolic compounds (100 μM), designated as follows: circles, phenol; triangles, Tyr; squares, VP-16; and diamonds, PMC. Other conditions were 10 μM H2O2, 10 μM H2O2, and 10 μM GSH. B, fluorescence responses were induced by the enzymes designated as follows: squares, cytochrome-c peroxidase-2; triangles, MPO; and circles, HRP. Reaction mixture contained 0.2 units/ml cytochrome-c peroxidase-2, 0.2 units/ml HRP, or 0.2 units/ml MPO and 50 μM phenol 10 μM H2O2, and 10 μM Ac-Tempo. GSH concentrations were 0 μM (closed symbols) and 10 μM (open symbols). a.u., arbitrary units.

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**Fig. 7.** Ac-Tempo-based detection of GS\(^{–}\) radicals in MPO-rich HL-60 cells incubated in the presence of H2O2/phenol. A, fluorescence microscopy. B, decay of Ac-Tempo EPR signal. C, fluorescence spectroscopy. Incubation conditions for fluorescence microscopy were as follows: a, 100 μM Ac-Tempo; b, same as a plus 100 μM phenol and 20 μM H2O2; c, same as b plus pre-treatment with 50 μM NEM; d, same as b plus pre-treatment with 0.4 mM succinyl acetone for 48 h; e, same as a plus phenol; and f, phenol and H2O2 without Ac-Tempo. Incubation time with reagents was 5 min. Incubation conditions for EPR spectroscopy and fluorescence spectroscopy were the same as described above, except that the Ac-Tempo concentration was 10 and 20 μM for EPR and fluorescence measurements respectively; the azide concentration was 2 mm. **, p < 0.05 versus cells treated with H2O2. ***, p < 0.05 versus cells treated with PhH2O2.

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present in control cell extracts after treatment with phenol/Ac-Tempo or H2O2/Ac-Tempo.

In separate experiments, the intracellular contents of Ac-Tempo and Ac-piperidine were quantitatively estimated by simultaneous EPR spectroscopy and fluorescence spectroscopy, respectively. These experiments confirmed a marked accumulation of MPO-catalyzed Ac-piperidine in cells. At the same time, a relatively slight decay of Ac-Tempo was observed suggesting that the plasma membrane is significantly less permeable to Ac-piperidine than to Ac-Tempo, thus allowing the latter to continuously enter cells due to its H2O2-dependent metabolism (Fig. 8A).
choline, phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine. No statistically significant difference was detectable between the major classes of PnA-labeled phospholipids extracted from either H$_2$O$_2$-treated cells or phenol-treated cells. Most importantly, Ac-Tempo prevented a H$_2$O$_2$/phenol-induced effect, implying that glutathionyl radicals play a key role in myeloperoxidase-catalyzed peroxidation of phospholipids. Moreover, Ac-Tempo protected HL-60 cells from phenol/H$_2$O$_2$-induced cytotoxicity (necrosis) despite the fact that the probe itself was slightly toxic (Fig. 10). These results indicate that glutathionyl radicals are likely involved in the mechanism of H$_2$O$_2$/phenol-induced cytotoxicity possibly mediated by phospholipid peroxidation.

**DISCUSSION**

Radical scavenging by antioxidants results in elimination of a reactive radical at the expense of the formation of a radical from an antioxidant molecule. In case of GSH, the scavenging of different reactive radicals yields sulfur-centered glutathionyl radicals, whose reactivity toward essential biomolecules determine whether GSH may function as an effective antioxidant or, on the contrary, participate in pro-oxidant propagation of free radical injury. To evaluate anti-oxidant/pro-oxidant properties of GSH, interactions of GS$^\cdot$ radicals with intracellular constituents need to be determined. Because GS$^\cdot$ radicals are formed as secondary radicals and often co-exist with the primary reactive radicals as well as with the subsequently produced free radical species such as GSSG$^\cdot$ and O$_2^\cdot$, these determinations are challenging under physiologically relevant conditions. This explains the lack of information on the interactions of GS$^\cdot$ radicals in cells.

Our approach to quantitatively assessing GS$^\cdot$ radicals was based on the use of Ac-Tempo, which undergoes two types of electron-exchange processes upon the reaction with GS$^\cdot$ radicals. In the first one, the chemical electron exchange reaction of Ac-Tempo with GS$^\cdot$ radicals forms a stable product, Ac-piperidine. In the latter, the second process, i.e. the physical electron exchange between the excited state of acridine and the nitroxide radical (which takes place in Ac-Tempo), is eliminated. Hence, instantaneously induced fluorescence from the acridine moiety permits the detection of GS$^\cdot$ radical formation.

Rosen and co-workers have demonstrated that massive production of O$_2^\cdot$ by neutrophils and xanthine/xanthine oxidase in the presence of thiols (cysteine) caused a high yield fluorescence emission from fluorophore-containing spin traps (16). Our results clearly demonstrate that peroxidase-induced oxidation of GSH by phenoxyl radicals is not dependent on superoxide generation. In particular, data on the effect of superoxide dismutase and spin traps in a model system containing HRP, phenol, H$_2$O$_2$, and GSH revealed that GS$^\cdot$ radicals were by far the most predominant contributors to the conversion of paramagnetic non-fluorescent Ac-Tempo into a non-paramagnetic fluorescent product that has been identified by our HPLC and MS analysis as Ac-piperidine (Scheme 1). In addition, our experiments with S-nitrosylated glutathione clearly demonstrated that conversion of Ac-Tempo into a non-paramagnetic fluorescent species occurs in the absence of peroxidase and reduced GSH via its direct interactions with GS$^\cdot$ radicals generated during the decomposition of GS-NO. These results also eliminate the potential modification of Ac-Tempo by peroxidase or by GSSG$^\cdot$ and O$_2^\cdot$ which could be formed in the reactions of glutathionyl radicals with GSH and oxygen.

In addition to the thyl radicals described here, other oxidants can potentially convert Ac-Tempo into a fluorescent form. However, we are aware of only one type of intracellular oxidants/radical relevant to this study that may potentially interact with Ac-Tempo. These are carbon-centered radicals that have been described as forming stable adducts with the nitroxide moiety of similar probes (27). However, we did not detect the formation of any adducts but did observe the formation of Ac-piperidine as the major product in the reaction catalyzed by purified horseradish peroxidase or myeloperoxidase in HL-60 cells. This suggests that carbon-centered radicals were not likely to contribute significantly to the fluorescence emission from the reaction products formed from Ac-Tempo.

GS$^\cdot$ radicals can trigger the formation of secondary radicals, including hydroxyl and peroxyl radicals. Hydroxyl radicals do not directly convert the probe into a fluorescent product. They can either oxidize methyl groups adjacent to the nitroxide moiety or oxidize nitroxide to oxo-ammonium cation (25), which, as a strong oxidant, is readily reduced back to nitroxide. Hydroxyl radicals, however, can convert the probe into a fluorescent product indirectly by attacking other biomolecules and generating carbon-centered radicals with the subsequent formation of stable adducts (16–18). As mentioned above, this was not the case either in the model system used or in HL-60 cells, because the adducts have not been detected.
Glutathionyl Radical-induced Oxidative Stress

GS radicals can facilitate the formation of peroxyl radicals. Previous work has shown that nitroxides catalyze conversion of peroxyl radicals into oxygen and alcohols (26). Nitroxides are not consumed during this reaction at all; hence, the probe cannot be converted to any fluorescent product by peroxyl radicals.

Thus, reactions between Tempo and other types of biologically relevant radicals (e.g. superoxide, hydroxyl, peroxyl, and carbon-centered radicals) do not yield piperidine as the major product (17, 25–27). It is possible that Ac-Tempo can react with other thiyl (e.g. cysteinyl) radicals; however, incomparably higher concentration of GSH renders it the major source of intracellular thiyl radicals. Overall, our current results, along with previously reported data, establish the specificity of the Ac-Tempo-based technique toward GS radicals.

Notably, exposure of HL-60 cells to H₂O₂ in the absence of phenolic compounds has been demonstrated to induce depletion of GSH mainly via the GSH-peroxidase pathway, which is not accompanied by any release of free GS radicals (28). In addition to GSH oxidation, H₂O₂ triggers the production of free radicals and the subsequent oxidative stress in different cells (including HL-60 cells) in the absence of phenolic compounds, yet H₂O₂ induced only a very weak fluorescence response in HL-60 cells. In contrast, phenolic compounds, which are usu-
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The depletion of endogenous ascorbate in a number of disease conditions with enhanced oxidative stress (e.g., those accompanied by inflammatory response) will inevitably create conditions for direct radical scavenging by GSH and other low molecular weight thiols that are capable of further propagating free radical damage.

Overall, it appears that an Ac-Tempo-based assay may provide interesting opportunities for specific and sensitive fluorescent imaging of intracellular sites of GS’ production as well as better understanding their biological effects. The importance of this possibility is underscored by the fact that the pronounced peroxidase activity of a number of enzymes (e.g., MPO, cyclooxygenase, and superoxide dismutase mutants) and the production of GS’ radicals may be associated with major disease conditions such as acute myeloid leukemia, amyotrophic lateral sclerosis, and gastric cancers (13, 20, 33, 34).

REFERENCES

1. Winterbourn, C. C. (1993) Free Radic. Biol. Med. 14, 85–90
2. Koppenol, W. H. (1993) Free Radic. Biol. Med. 14, 91–94
3. Sturgeon, B. E., Sipe, H. J., Jr., Barr, D. P., Corbett, J. T., Martinez, J. G., and Mason, R. P. (1998) J. Biol. Chem. 273, 30116–30121
4. Ford, E., Hughes, M. N., and Wardman, P. (2002) Free Radic. Biol. Med. 32, 1314–1323
5. Galati, G., Sabzevari, O., Wilson, J. X., and O’Brien, P. J. (2002) Toxicology 177, 91–104
6. Pereski, D., and Schoneich, C. (2001) Free Radic. Biol. Med. 31, 98–107
7. Zhao, R., Lind, J., Merbnyi, G., and Erikson, T. E. (1994) J. Am. Chem. Soc. 116, 1210–12105
8. Schoneich, C., Dillinger, U., Von Bruchhausen, F., and Asmus, K. D. (1992) Arch. Biochem. Biophys. 292, 456–467
9. Sprinz, H., Schwinn, J., Naumov, S., and Brede, O. (2000) Biochim. Biophys. Acta 1483, 91–100
10. Ferreri, C., Costantino, C., Perrutta, L., Landi, L., Mulazzani, Q. G., and Kagan, V. E. (1999) Arch. Biochem. Biophys. 368, 769–784
11. Kagan, V. E., Yalowich, J. C., Banni, S., Day, B. W., Claycamp, H. G., and Kagan, V. E. (1996) Arch. Biochem. Biophys. 330, 3–11
12. Kwan, H. S., Yun, H. S., Cheok, P. B., and Yun, M. B. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4525–4526
13. Kagan, V. E., Yalowich, J. C., Banni, S., Day, B. W., Claycamp, H. G., and Kagan, V. E. (1996) Arch. Biochem. Biophys. 330, 3–11
14. Pou, S., Huang, Y. I., Bhan, A., Bhadti, V. S., Hosmane, R. S., Wu, S. Y., Cao, G. L., and Rosen, G. M. (1993) Anal. Biochem. 212, 85–90
15. Blough, N. V., and Simpson, D. J. (1988) J. Am. Chem. Soc. 110, 1915–1917
16. Herbelin, S. E., and Blough, N. V. (1998) J. Phys. Chem. B 102, 8170–8176
17. Ross, D., Albano, E., Nilsson, U., and Meldeus, P. (1984) Biochim. Biophys. Acta 781, 159–165
18. Villegas, I., Martin, M. J., La Casa, C., Motivia, V., and De La Lastra, C. A. (2002) Free Radic. Res. 36, 769–777
19. Rito, V., Banni, S., Yalowich, J. C., Day, B. W., Claycamp, H. G., Corongiu, F. P., and Kagan, V. E. (1996) Biochim. Biophys. Acta 1283, 127–140
20. Chalvardjian, A., and Rudnicki, E. (1970) Arch. Biochem. Biophys. 146, 524–530
21. Burgener, U., and Ohlinger, C. (1997) FEBS Lett. 411, 269–274
22. Samuni, A., Goldstein, S., Russo, A., Mitchell, J. B., Krishna, M. C., and Neta, P. (2002) J. Am. Chem. Soc. 124, 8719–8724
23. Barton, H. R., Le Gloahec, V. N., and Smith, J. (1998) Tetrahedron Lett. 39, 7483–7486
24. Zhang, R., Goldstein, S., and Samuni, A. (1999) Free Radic. Biol. Med. 26, 1245–1252
25. Harman, L. S., Carver, D. K., Schreiber, J., and Mason, R. P. (1986) J. Biol. Chem. 261, 1622–1628
26. Rosen, G. M., and Rauchman, E. (1977) Biochim. Biophys. Acta 519, 115–119
27. Sarre, D. F., and Kennedy, D. B. (1992) J. Biol. Chem. 267, 20384–20400
28. Kapiotis, S., Sengoegeles, G., Herrmann, M., Held, I., Seelos, C., and Gmeiner, B. M. (1997) Arterioscler. Thromb. Vasc. Biol. 17, 2855–2860
29. Day, B. W., Tsuji, Y., Tsuji, Y., Liao, M., Fahey, J. A., Carta, G., Klein, E. R., Dube, R. K., and Kagan, V. E. (1990) Chem. Res. Toxicol. 12, 28–37
30. Zhang, H., Joseph, J., Gurney, M., Becker, D., and Kalyanaraman, B. (2002) J. Biol. Chem. 277, 2013–2020
31. Schoneich, C., Leung, W. K., Ju, M. Y., To, K. F., Cheng, A. S., Ng, E. K., and Chan, F. K. (2000) Am. J. Pathol. 157, 729–735

Finally, very good substrates for heme-containing peroxidases, can switch H2O2 metabolism from a safe GSH peroxidase-catalyzed pathway that results in the formation of GSSG to a myeloperoxidase-catalyzed utilization of H2O2 that induces the production of GS radicals. Importantly, the amount of GSH oxidized in HL-60 cells in the presence of phenol and Ac-Tempo was equal to the amount of Ac-Tempo converted into Ac-piperidine. These observations demonstrate that glutathionyl radicals were absolutely dominant in the fluorescence response of Ac-Tempo, whereas other radical species were only insignificant contributors.

Non-specific reduction of the nitroxide moiety of Ac-Tempo by intracellular reductants (e.g. by membrane electron transporting proteins) can occur in cells (29). To assure that the non-specific reduction of Ac-Tempo in HL-60 cells did not interfere with the assay, we performed control experiments and found that the rate of fluorescence production was approximately two orders of magnitude higher in the presence of phenol and H2O2 (i.e. during myeloperoxidase reaction) than in their absence. In line with this, the Ac-Tempo reduction product, Ac-Tempo hydroxylamine, was not detectable in HL-60 cells by either MS or HPLC analysis. In addition, the fluorescence intensity from cells with high MPO activity was manyfold higher than in cells pre-treated with myeloperoxidase inhibitors, succinyl acetone, or azide. Finally, we performed several tests for the potential Ac-Tempo reduction by GSH in cell-free model systems. We were unable to detect any reduction of the probe by GSH in the concentration range from 0.01 to 5 mM. Thus, direct reduction of Ac-Tempo by glutathione is unlikely. Therefore, this report is the first to demonstrate imaging of GS radicals in cells with stimulated MPO activity.

Using cells with metabolically Pan-labeled phospholipids, we found that stimulation of MPO activity with phenol and H2O2 caused significant oxidation of all major classes of phospholipids. Because both phenoxyl radicals and GS radicals (the latter generated via interaction of the former with intracellular GSH) are massively produced during incubation of MPO-rich cells with H2O2/phenol, it has not been established which of these is involved in phospholipid peroxidation. Both phenoxyl radicals and GS radicals are strong oxidizing radicals (with the redox potentials ~900 mV) capable of oxidizing phospholipids. Previous work has identified phenoxyl radicals generated from phenol or tyrosine as a free radical species involved in the peroxidation of lipids in simple liposomal model systems or low density lipoproteins (30–32). Based on the herein established specificity of Ac-Tempo toward GS radicals (but not toward phenoxyl radicals, or oxygen radicals), our results, which demonstrate the complete inhibition of H2O2/phenol-induced phospholipid oxidation by Ac-Tempo paralleled by a significant inhibition of GSH oxidation, strongly suggest that glutathionyl radicals were mostly responsible for the effect in cells. It is likely that very high intracellular concentrations of GSH make it more vulnerable to a direct attack by phenoxyl radicals than by phospholipids. Thus, using Ac-Tempo, we demonstrated for the first time that GS radicals can induce phospholipid peroxidation and cause cytotoxicity during H2O2-supported MPO-catalyzed metabolism of phenol in HL-60 cells. In tissues and biological fluids, endogenous ascorbate acts as the primary scavenger of phenoxyl radicals, thus preventing their direct interactions with GSH and the propagation of oxidative stress.
Glutathione Propagates Oxidative Stress Triggered by Myeloperoxidase in HL-60 Cells: EVIDENCE FOR GLUTATHIONYL RADICAL-INDUCED PEROXIDATION OF PHOSPHOLIPIDS AND CYTOTOXICITY
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J. Biol. Chem. 2004, 279:23453-23462.
doi: 10.1074/jbc.M400119200 originally published online March 23, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M400119200

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