Detection of Glutathione Thyl Free Radical Catalyzed by Prostaglandin H Synthase Present in Keratinocytes

STUDY OF COOXYDATION IN A CELLULAR SYSTEM*

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We report here the application of the electron spin resonance technique to detect free radicals formed by the hydroperoxidase activity of prostaglandin H synthase in cells. Studies were done using keratinocytes obtained from hairless mice. These cells can be prepared in large number and possess significant prostaglandin H synthase activity. Initial attempts to directly detect free radical metabolites of several amines in cells were unsuccessful. A technique was developed based on the ability of some free radicals formed by prostaglandin hydroperoxidase to oxidize reduced glutathione (GSH) to a thyl radical, which was trapped by 5,5-dimethyl-1-pyrroline N-oxide (DMPO). Phenol and aminopyrine are excellent hydroperoxidase substrates for this purpose and thus were used for all further experiments. Using this approach we detected the DMPO/GS’ thyl radical adduct catalyzed by cellular prostaglandin hydroperoxidase. The formation of the radical was dependent on the addition of substrate, inhibited by indomethacin, and supported by either exogenous arachidonic acid or endogenous arachidonic acid released from phospholipid stores by Ca2+ ionophore A-23187. The addition of GSH significantly increased the intracellular GSH concentration and concomitantly stimulated the formation of the DMPO/GS’ thyl radical adduct. Phenol, but not aminopyrine, enhanced thyl radical adduct formation and prostaglandin formation with keratinocytes while both cofactors were equally effective in incubations containing microsomes prepared from keratinocytes. These results suggest that prostaglandin hydroperoxidase-dependent co-oxidation of chemicals can result in the intracellular formation of free radical metabolites.

Prostaglandin H synthase catalyzes the conversion of arachidonic acid to prostaglandin endoperoxides. The enzyme possesses two distinct enzymatic activities: the fatty acid cyclooxygenase converts arachidonic acid to the hydroperoxy endoperoxide prostaglandin G3, whereas the hydroperoxidase reduces the hydroperoxy group of prostaglandin G2 to the corresponding alcohol, forming prostaglandin H2 (1-3). A large number of chemicals, including some carcinogens, supply reducing equivalents to the hydroperoxidase and undergo subsequent oxidation during this reduction of prostaglandin G2 to prostaglandin H2. This oxidation is dependent on the formation of the hydroperoxy endoperoxide prostaglandin G2, although other peroxides such as H2O2 or 15-hydroperoxyeicosatetraenoic acid will also support the oxidation of these chemicals (1-4).

In supplying reducing equivalents to the hydroperoxidase, many reducing cofactors are oxidized to free radical intermediates in accordance with the classical horseradish peroxidase mechanism (5). The formation of free radical metabolites by prostaglandin H synthase was detected with either direct ESR or the spin-trapping technique using in vitro incubation systems. The formation of aminopyrine (6, 7), benzoic acid, and tetramethylbenzidine cation-free radicals (9), the sulfur trioxide anion free radical (10), and DMPO/GS’ thyl free radical (11) by prostaglandin H synthase present in mammary gland vesicle microsomes has been detected by ESR. The detection of free radical metabolites by the reduction of toxic chemicals using intact cellular systems has been reported for nitromidazoles (12) and gentian violet (13). Direct demonstration of the existence of cellular free radical metabolites formed by prostaglandin H synthase or other mammalian peroxidases has not been reported, although indirect evidence for their existence in vivo is available. Attempts to detect directly with ESR free radical metabolites formed by prostaglandin hydroperoxidase present in intact cells are, in general, hindered by low enzymatic activity of cells compared with mammary gland vesicle microsomes, the limited number of cells available, the inherent instability of the free radicals, and the presence of endogenous reducing cofactors present in intact cells.

In this paper, we report the detection of free radical metabolites by ESR techniques using a cellular system. The mouse keratinocytes were selected as the cellular system since they can be easily prepared in large numbers, possess relatively high prostaglandin H synthase activity (14), and are low in the endogenous reducing agent, ascorbate, which could reduce the spin adduct to a diamagnetic moiety and, thus, render ESR detection impossible.

EXPERIMENTAL PROCEDURES

Chemicals—5,5-Dimethyl-1-pyrroline N-oxide was purchased from Aldrich, phenol, reduced glutathione, indomethacin, glycine, protease, DNase, and aminopyrine from Sigma, unlabeled arachidonic acid

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§ The abbreviations used are: DMPO, 5,5-dimethyl-1-pyrroline N-oxide; GSH, reduced glutathione; HEPES, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; HPBS, HEPES phosphate-buffered saline; HPLC, high pressure liquid chromatography; Me2SO, dimethyl sulfoxide.
from NuChek Prep, Inc. (Elysian, MN). [14C]prostaglandin E2, [3H]5,6,8,9,11,12,14,15-arachidonic acid, [3H]phenol, and [3H]gluta-
ephone were obtained from New England Nuclear and Ca2+ ionophore A-23187 from Calbiochem (La Jolla, CA). DMPO was used after puri-
fication with charcoal (15). Percoll was obtained from Pharmacia
LKB Biotechnology Inc. All other reagents were of the highest purity
commercially available and were used without further purification.

The isolation and the preparation of the keratinocytes were as follows: HPBS (150 mM NaCl, 5 mM KCl, 4 mM KH2PO4, 5.5 mM
sucrose, 25 mM HEPES, pH 7.4) and media (Krebs-Henseleit containing
5% bovine serum albumin with 0.12% trypsin inhibitor, 0.0485%
deyoxyribonuclease I, 0.018% CaCl2).

Percoll gradients and terminal vesicle microsomes were prepared as
described previously (16). Protein concentrations were determined by
the Lowry assay (17).

The keratinocytes were isolated using a procedure developed by
Coomes et al. (18). Briefly, the skin from HRS/J female mice (Charles
River), 6-8 weeks old, was obtained after killing the animals by
cervical dislocation. The epidermis was separated from the dermis by
enzymatic digestion with collagenase and gentle stirring in 0.1% trypsin
solution. The epidermis was broken into individual cells by shaking it with
and gentle stirring in 0.1% trypsin. The cells were filtered sequentially through
160- and 40-μm silk mesh, then centrifuged for 10 min at 1500 rpm
at 4°C in a J6-B Beckman centrifuge. The cells were washed twice
and resuspended in HPBS with 0.0485% Dnase. Cell number was
determined on a hemocytometer and cell viability was 80-90% as
determined using trypan blue exclusion. In some experiments the epidermis
was disrupted by a polytron (20 s at 0°C) and the microsomal fraction
prepared by differential ultracentrifugation.

ESR Measurements—ESR spectra were recorded with a Varian E-
109 spectrometer in a flat cell mounted in a TM10 cavity. A modula-
tion frequency of 100 kHz was used, and all spectra were recorded
with a microwave power of 20 milliwatts, a scan range of 8 millitesla
(80 G), a scan time of 2-4 min, and a time constant of 1 s. Receiver
gain and modulation amplitude are given in the legends to the figures.
All investigations were done at room temperature in 100 mM phos-
phate buffer, pH 7.4. In all experiments the reaction was quenched after
a 10% final concentration, and the pH was adjusted to 3.5 with acetic
acid. The aqueous solution was applied to a Prep-Sep (Waters Assoc.,
Milford, MA), the column washed with 7.5 ml of water and  then
washed several times with phosphate buffer and dissolved in 0.1
HPBS and Percoll solutions were aspirated away, the pelleted cells
(1-1.2 × 106) resuspended in 0.4 ml of fresh HPBS (without [3H]
GSH or glycine), counted in a hemocytometer, sonicated, and counted
to determine radioactivity in a liquid scintillation spectrometer. The initial
content of GSH in these cellular suspensions was determined using
the enzymatic method of Tietze (22).

RESULTS

Initial Cell Experiments—Previous studies on the formation of
cation-free radicals by prostaglandin hydroperoxidase sug-
gested that tetramethylbenzidine (9) or aminopyrine (6, 7) would be suitable
prostaglandin hydroperoxidase substrates for detection of these radicals using intact cells. Keratinocytes
were incubated with these chemicals under various conditions
including the addition of arachidonic acid. However, we could
not detect free radical metabolites of either tetramethylben-
zidine or aminopyrine under any conditions. Previous studies
by our laboratory (7), as well as others (23), showed that
amino free radicals from tetramethylbenzidine or aminopy-
rine are reduced to the parent amine by GSH, which is present
in cells, with concurrent oxidation of GSH to a thyl radical:

\[ \text{AR} + \text{GSH} \rightarrow \text{GSSG} + \text{AR} \]

The thyl radical, GS', can be trapped by DMPO and easily
detected with ESR in vitro (24). Reoxidation of the amine
and its eventual reduction by GSH can result in the formation of
the thyl radical with a resultant magnification in the
enzymatic thyl radical formation (7). Spin trapping by
DMPO of thyl radicals formed as a result of an initial
oxidation of a substrate may be a useful technique for the
determination of free radical formation by prostaglandin hy-
droperoxidase in cells, where GSH will prevent the accumu-
lation of detectable concentrations of substrate radicals.

In Vitro Experiments—When ram seminal vesicle micro-
somes were incubated with phenol, GSH, arachidonic acid,
and DMPO, a four-line ESR spectrum (Fig. 1A) with hyper-
fine splitting constants of \( a^N = 1.53 \) millitesla (15.3 G) and
\( a^G = 1.62 \) millitesla (16.2 G) was detected, which is character-
istic for the DMPO/ GS' thyl radical adduct (24). The addi-
tion of the cyclooxygenase inhibitor indomethacin (10 μM)
hindered by 90% the prostaglandin H synthase activity as
measured by oxygen incorporation (data not shown) and the formation
of the DMPO/ GS' thyl radical adduct (B). The
addition of H2O2 in place of arachidonic acid also supported thyl
radical adduct signal indicating the involvement of pros-
taglandin hydroperoxidase (C). The addition of indomethacin
incubations containing H2O2 but no arachidonic acid did
not suppress thyl radical adduct formation (D). The omission

Prostaglandin H Synthase-dependent Thiyl Free Radical Formation in Cells
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Component of the system and concentrations used: 30 mM DMPO, 0.125 mg/ml ram seminal vesicle microsomes, 0.5 mM phenol, 0.5 mM GSH, 0.1 mM arachidonic acid (AA). ESR parameters: scan range 8 millitesla (mT), scan time 2 min, gain $6.3 \times 10^4$, modulation amplitude 0.26 millitesla, time constant 0.5 s. A, complete system (ram seminal vesicle microsomes, DMPO, phenol, GSH, arachidonic acid) showing the ESR signal of the DMPO/GS' thiyl radical adduct ($a_N = 1.53$ millitesla, $a_H = 1.62$ millitesla). B, as A with 10 μM indomethacin (INDO). C, as A without arachidonic acid plus H$_2$O$_2$ (100 μM). D, as A with 10 μM indomethacin and 50 μg catalase (CAT). E, as A without arachidonic acid plus catalase (50 μg). F, as A without arachidonic acid, with 10 μM indomethacin and 100 μM H$_2$O$_2$. G, as A without GSH. H, as A without enzyme.

These results indicate that although GSH is a substrate for the hydroperoxidase, the addition of phenol, a better substrate for the hydroperoxidase, greatly magnifies the formation of the thiyl radical. The aromatic amine aminopyrine also mag-

![Fig. 1. DMPO spin trapping of the thiyl radical in a system containing ram seminal vesicle microsomes and phenol. Components of the system and concentrations used: 30 mM DMPO, 0.125 mg/ml ram seminal vesicle microsomes, 0.5 mM phenol, 0.5 mM GSH, 0.1 mM arachidonic acid (AA). ESR parameters: scan range 8 millitesla (mT), scan time 2 min, gain $6.3 \times 10^4$, modulation amplitude 0.26 millitesla, time constant 0.5 s. A, complete system (ram seminal vesicle microsomes, DMPO, phenol, GSH, arachidonic acid) showing the ESR signal of the DMPO/GS' thiyl radical adduct ($a_N = 1.53$ millitesla, $a_H = 1.62$ millitesla). B, as A with 10 μM indomethacin (INDO). C, as A without arachidonic acid plus H$_2$O$_2$ (100 μM). D, as A with 10 μM indomethacin and 50 μg catalase (CAT). E, as A without arachidonic acid plus catalase (50 μg). F, as A without arachidonic acid, with 10 μM indomethacin and 100 μM H$_2$O$_2$. G, as A without GSH. H, as A without enzyme.](image1)

![Fig. 2. DMPO spin trapping of the thiyl radical in a system containing ram seminal vesicle microsomes but no phenol. Components of the system and concentrations used: 30 mM DMPO, 0.125 mg/ml ram seminal vesicle microsomes, 0.5 mM GSH, 0.1 mM arachidonic acid (AA). ESR parameters: scan range 8 millitesla (mT), scan time 4 min, gain $6.3 \times 10^4$, modulation amplitude 0.13 millitesla, time constant 1 s. A, complete system (ram seminal vesicle microsomes, DMPO, GSH, arachidonic acid) showing the DMPO/GS' thiyl radical adduct. B, as A with 10 μM indomethacin (INDO). C, as A without arachidonic acid. D, as A with 100 μM indomethacin and 50 μg catalase (CAT). E, as A without arachidonic acid plus catalase (50 μg). F, as A without arachidonic acid, with 10 μM indomethacin and 100 μM H$_2$O$_2$. G, as A without GSH. H, as A without enzyme.](image2)
nified the microsomal-dependent formation of the DMPO/ GS' thiyl radical adduct (see below).

*Cell Experiments*—Incubations of mouse keratinocytes with phenol, GSH, arachidonic acid, and the spin trap DMPO gave the characteristic four-line pattern of the DMPO/GS' thiyl radical adduct (Fig. 3A). The addition of 0.6% ethanol, the solvent for indomethacin, brought the total ethanol concentration to 1.2% and reduced the signal size to about 50% of that of the complete system (B). Nearly complete inhibition was achieved with 100 μM indomethacin, indicating prostaglandin H synthase cyclooxygenase involvement in the formation of the DMPO/GS' thiyl radical adduct (C). No DMPO/GS' thiyl radical adduct was observed in the absence of cells (D) or arachidonic acid (E), which is consistent with the high degree of inhibition by indomethacin in Fig. 3C. An incubation without the addition of GSH gave a smaller, but unmistakable signal of the DMPO/GS' thiyl radical adduct, apparently derived from endogenous GSH (F). In the absence of phenol (G), a small adduct signal was observed which was due either to a direct cooxidation of GSH by prostaglandin hydroperoxidase or to oxidation of other cellular substrates which, in turn, would then oxidize GSH to its thiyl radical.

In order to further investigate the dependence of the DMPO/GS' thiyl radical adduct formation on arachidonic acid and endogenous GSH, experiments were done in which Ca2+ ionophore A-23187 was employed to release endogenous arachidonic acid from phospholipid stores, or in which exogenous GSH was omitted. ESR spectra obtained from a standard incubation consisting of the cells, phenol, GSH, arachidonic acid, and the spin trap DMPO is shown in Fig. 4A. In the absence of added GSH, a smaller DMPO/GS' thiyl radical adduct signal was observed (B), indicating that endogenous levels of GSH in intact cells are sufficient for formation of the free radical. In the absence of added arachidonic acid, but in the presence of added GSH, the release of endogenous arachidonic acid by Ca2+ ionophore A-23187 (10 μM in Me2SO) also produced the DMPO/GS' thiyl radical adduct (C). The ESR spectrum obtained with the vehicle Me2SO (D), shows little or no thiyl radical signal which indicates there is little free arachidonic acid available in the absence of stimulation of phospholipase activity.

*Phenol Metabolites*—The proposed mechanism by which phenol enhances thiyl radical formation (Scheme 1) obligates the existence of the phenoxyl radical. The phenoxyl radical, however, cannot be detected by direct ESR under our experimental conditions, presumably because this reactive free radical does not reach detectable levels. An attempt was made to detect free radical-mediated metabolites of phenol such as o,o'-biphenol or p,p'-biphenol. Horseradish peroxidase or myeloperoxidase oxidizes phenol to these biphenols (25). The subsequent oxidation of biphenols results in the formation of conjugates with GSH as well as binding to protein. [14C] Phenol was oxidized to both o,o'-biphenol and p,p'-biphenol by ram seminal vesicle microsomes in the presence of arachidonic acid (Table I). The metabolites were assigned on the

![Fig. 3](image-url)

**Fig. 3.** DMPO spin trapping of the thiyl radical in a system containing mouse keratinocytes. Components of the system and concentrations used: cells (10^7 cells/ml), 30 mM DMPO, 0.5 mM phenol, 0.5 mM GSH, 0.1 mM arachidonic acid. ESR parameters: scan range 8 millitesla (mT), scan rate 2 millitesla/min, gain 3.2 × 10^5, modulation amplitude 0.133 millitesla, time constant 1 s. A, complete system (keratinocytes, DMPO, phenol, GSH, and arachidonic acid) showing the DMPO/GS' thiyl radical adduct signal. B, as A with additional (0.6%) ethanol (final conc. 1.2%). C, as A with 0.1 mM indomethacin (final conc. of ethanol 1.2%). D, as A without cells. E, as A without arachidonic acid. F, as A without GSH. G, as A without phenol. The ESR spectra was recorded approximately 4 min after initiation of the reaction.

![Fig. 4](image-url)

**Fig. 4.** DMPO spin trapping of the thiyl radical in a system containing keratinocytes and Ca2+ ionophore A-23187. Components of the system and concentrations used: cells (10^7 cells/ml), 30 mM DMPO, 0.5 mM phenol, 0.5 mM GSH, 0.1 mM arachidonic acid. ESR parameters: scan range 8 millitesla (mT), scan rate 2 millitesla/min, gain 2 × 10^5, modulation amplitude 0.133 millitesla, time constant 1 s. A, complete system (cells, DMPO, phenol, GSH, and arachidonic acid) showing the DMPO/GS' thiyl radical adduct. B, as A without GSH. C, as A without arachidonic acid, but with Ca2+ ionophore A-23187 (10 μM dissolved in Me2SO). D, as A without arachidonic acid, with Me2SO.
basis of coelution with authentic, nonradioactive standards which were monitored by UV absorption. The p,p'-biphenol was the major metabolite detected. Oxidation was dependent on arachidonic acid and protein, and inhibited by the addition of indomethacin to the incubation (data not shown). When GSH was added to the incubation mixtures, both biphenolic product formation as well as the covalent binding of radiolabel to protein were significantly reduced. Moreover, the amount of [14C]phenol not extracted from aqueous solution that is unmetabolized phenol; this indicates that GSH reduced the 0.41 ± 0.02 nmol/10^6 cells (22). GSH uptake is quite rapid as there was a substantial increase in intracellular GSH (approximately 0.15 nmols/10^6 cells) at the earliest time point, 3 min, which coincides with the recording of the ESR spectra in Figs. 3 and 4.

**Glutathione Uptake**—In experiments designed to measure the uptake of GSH into keratinocytes, it was observed that intracellular GSH-associated radioactivity increased with time in four different trials (Fig. 5). At 30 min from the addition of 1 mM extracellular [3H]GSH, the increase in intracellular [3H]GSH ranged from 0.25 to 0.37 nmol/10^6 cells.

That this increase was due to GSH and not to other derivatives of GSH was ensured as a diversionary substrate (glycine) was provided for the GSH-degrading enzymes (20). Also the GSH was dissolved and added immediately to the suspensions to minimize GSH oxidation. That the increase in GSH with time was due to uptake of GSH (a saturable process) and not due to exchange (a nonsaturable process) was confirmed as the radioactivity content of a sample taken at a later time (75 min) was unchanged from the 30-min time point taken from that same suspension. In separate experiments the amounts of GSH in the cells at the time of isolation was determined to be 0.41 ± 0.02 nmol/10^6 cells (22). GSH uptake is quite rapid as there was a substantial increase in intracellular GSH (approximately 0.15 nmols/10^6 cells) at the earliest time point, 3 min, which coincides with the recording of the ESR spectra in Figs. 3 and 4.

**Cellular Versus Microsomal Formation of Thiyl Radical**—Several aromatic amines including aminopyrine significantly enhanced DMPO/GS' thiyl radical adduct formation (data not shown) as observed with phenol in studies using ram seminal vesicle microsomes as the source of prostaglandin H synthase. A comparison between phenol and aminopyrine in their ability to enhance thiyl radical formation by ram seminal vesicle microsomes is shown in Fig. 6. The intensity of the DMPO/GS' thiyl radical adduct, estimated from the third line of the ESR spectrum, was dependent on the protein concentration. Under identical incubation conditions, the relative intensity of the ESR signal was similar with phenol and aminopyrine. At microsomal concentrations greater than 0.1 mg/ml, aminopyrine yielded a higher DMPO/GS' thiyl radical adduct signal intensity than did phenol. These results suggest that aminopyrine, like phenol, should magnify formation of the DMPO/GS' thiyl radical adduct to a similar extent in the cellular system.

**ESR spectra from incubations of mouse keratinocytes with**
either phenol or aminopyrine, GSH, arachidonic acid, and the spin trap DMPO are shown in Fig. 7. Incubations with phenol gave the characteristic four-line pattern of the DMPO/GS' thiyl radical adduct (Fig. 7A). However, little or no detectable DMPO/GS' thiyl radical adduct was observed when aminopyrine rather than phenol was used (Fig. 7B). Thus, in contrast to the results from ram seminal vesicle microsomal incubations (Fig. 6), addition of aminopyrine to keratinocytes did not enhance the DMPO/GS' thiyl radical adduct formation as observed for phenol.

**To support the results of our ESR experiments, we analyzed arachidonic acid metabolites formed by the keratinocytes and**

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**TABLE 1**

| Incubation       | Experiment no. | Phenol | o,o'-Biphenol | p,p'-Biphenol | Conjugated | Protein bound |
|------------------|----------------|--------|---------------|---------------|------------|---------------|
| Complete*        | 1              | 388    | 5.1           | 23.5          | 3.6'       | 9.6           |
|                  | 2              | 346    | 6.9           | 50.1          | 6.0        | 12.3          |
| Complete + GSH*  | 1              | 454    | ND            | 4.8           | 23.1       | 2.4           |
|                  | 2              | 489    | ND            | 17.1          | 18.5       | 2.4           |
| Complete - arachidonic acid | 1 | 500   | ND            | 2.4           | ND         | 0.3           |
|                  | 2              | 496    | ND            | 5.7           | ND         | 0.6           |

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* Incubations contained in 1.0 ml of buffer, 0.5 mg/ml ram seminal vesicle microsomes, 100 μM arachidonic acid, and 500 μM [14C]phenol, and were incubated and processed as described in the text.

* All values are nmol from each of two separate experiments.

* Water-soluble, nonprotein bound. These and protein bound values were corrected for nonspecific binding values obtained with boiled microsomes.

* 0.5 mM GSH.

* Not detectable.
Fig. 5. Uptake of [3H]glutathione by isolated keratinocytes.
Uptake of GSH by intact epidermal keratinocytes; four separate trials are shown. Cells were incubated at 37°C in modified HPBS (see text). At time 0 [3H]GSH (1 mM final at a specific activity from 1500-2500 dpm/nmol) was added. The first time point, actually taken at time 0 (i.e. the time of addition of [3H]GSH), is indicated on the figure at 3 min, as this was the time that elapsed before the cells were layered on and spun through the underlying Percoll solution. In trials where HPBS containing just [3H]GSH was spun atop the Percoll, no radioactivity was detected in the bottom of the tubes. In one trial, an aliquot was taken at 75 min and is indicated by the broken line.

Fig. 6. Comparison of phenol to aminopyrine on the enhancement of DMPO/GS' thiyl radical adduct formation with ram seminal vesicle microsomes. The components of the system and the ESR parameters were identical to those used with the intact cells, both phenol and aminopyrine concentrations were 0.5 mM. The ESR parameters were scan range 8 millitesla, modulation amplitude 0.133 millitesla, time constant 0.5 s. The relative intensity of the DMPO/GS' thiyl radical adduct was estimated from the height of the third line.

DISCUSSION
Prostaglandin H synthase converts arachidonic acid to prostaglandins. Keratinocytes were incubated with [3H]arachidonic acid in the presence or absence of phenol or aminopyrine (500 µM), with [3H]arachidonic acid (100 µM), for 5 min. Prostaglandins were isolated and analyzed by HPLC as described under "Experimental Procedures." Values are mean ± S.D. N = 3-4.

Significantly different from incubations lacking cofactor. p < 0.05 by Fisher's least squares difference.

Pyrine should enhance the conversion of arachidonic acid to prostaglandins. Keratinocytes were incubated with [3H]arachidonic acid in the presence or absence of aminopyrine or phenol. Conversion of arachidonic acid to prostaglandins was estimated by HPLC analysis. As shown in Table II, the addition of phenol to the keratinocytes enhanced prostaglandin formation by approximately 2-fold. In contrast, aminopyrine under identical incubation conditions did not enhance prostaglandin formation. The addition of the spin trap DMPO did not alter arachidonic acid metabolism (data not shown). Thus, phenol, but not aminopyrine, enhanced both DMPO/GS' thiyl radical adduct formation and prostaglandin formation with intact keratinocytes.

In further experiments, keratinocytes were disrupted and a microsomal fraction was prepared and used as a source of prostaglandin hydperoxidase. The keratinocyte microsomes were incubated with [3H]arachidonic acid in the presence or absence of phenol or aminopyrine. Under incubation conditions identical to those used with the intact cells, both phenol and aminopyrine enhanced prostaglandin formation as shown in Table II by approximately 3-fold. Thus, both DMPO/GS' thiyl radical adduct formation and prostaglandin formation are enhanced to a similar extent by peroxidase substrates with the keratinocyte microsomal system.
incorporation of 2 mol of $O_2/mol$ of arachidonic acid, thereby forming the hydroperoxy endoperoxide, prostaglandin G$_2$. If a reducing cofactor is present, this hydroperoxide is reduced to the hydroxy compound prostaglandin H$_2$ by the hydroperoxidase activity of prostaglandin H synthase with the concomitant oxidation of the reducing cofactor (1–3). Because the hydroperoxidase metabolism is a one-electron oxidation, intermediates of the reaction are free radicals which have been detected either directly with ESR (6, 7, 9) or indirectly with the spin-trapping technique (7, 24). These studies have been limited to the use of in vitro conditions using either microsomes or purified enzyme.

This work provides evidence that free radicals formed in the metabolism of chemicals by the prostaglandin H synthase/arachidonic acid system are generated and detected in a cellular system. A free radical was detected using a technique that magnifies the formation and steady-state concentration of the DMPO/GS' thiyl radical adduct. The technique depends on the initial oxidation of a co-oxidizable substrate by prostaglandin hydroperoxidase to a free radical and the subsequent oxidation of GSH to a thiyl radical by the initially formed free radical (Scheme 1). We propose the reaction sequence shown, in which arachidonic acid is metabolized to prostaglandin G$_2$ by the cyclooxygenase activity of prostaglandin H synthase. Prostaglandin G$_2$, in turn, is reduced by the hydroperoxidase to prostaglandin H$_2$, with phenol being oxidized to the phenoxyl radical. The phenoxyl radical is then reduced, thereby oxidizing GSH to GS', which we detected by spin trapping with DMPO. The reaction sequence is analogous to the reaction shown for the aminopyrine-free radical and subsequent thiyl radical formation (7). At the completion of our proposed reaction sequence (Scheme 1) the reduction of the phenoxyl radical by GSH would regenerate phenol which may then re-enter the reaction sequence. According to this reaction sequence, phenol would greatly enhance the formation of thiyl radical.

In conformance with our reaction sequence, phenol greatly enhanced the formation of thiyl radical in ram seminal vesicle microsomes (Fig. 1) and in keratinocytes (Fig. 3). According to Scheme 1, increased thiyl radical formation would obligate increased arachidonic acid metabolism by prostaglandin H synthase. Conforming again to Scheme 1, we demonstrated increases in arachidonic acid metabolism in keratinocytes upon addition of phenol (Table II) as has been previously shown with microsomal systems by Egan et al. (26). This indicates phenol is oxidized, supplying reducing equivalents to prostaglandin H synthase. Thus, two mechanisms exist for the stimulation of DMPO/GS' thiyl radical adduct formation by the addition of phenol to cells, increasing arachidonic acid oxidation and co-oxidation of a better substrate, phenol.

While there is no direct evidence for the involvement of the phenoxyl free radical in the mouse skin cell system, biphenolic products, which are derived from the phenoxyl free radical, could be detected in the ram seminal vesicle microsomal system (Table I). From Scheme 1 it is predicted that GSH would reduce the phenoxyl radical to phenol, thereby decreasing the biphenolic products and, concomitantly, increasing the level of phenol. Again our results conform to Scheme 1, as exogenous GSH both decreased the formation of the biphenolic products and increased the level of phenol (Table I).

The formation of thiyl radical in the cells was arachidonic acid-dependent and indomethacin-inhibitable, thus pointing to the role of the cyclooxygenase. The reaction in cells was also initiated by the release of endogenous arachidonic acid by the Ca$^{2+}$ ionophore A-23187. The signals obtained were smaller than those obtained with the addition of 100 $\mu$M arachidonic acid, probably due to the slow release of endogenous arachidonic acid from the membrane phospholipids compared with the addition of exogenous arachidonic acid. The height of the DMPO/GS' thiyl radical adduct ESR signal is a steady-state quantity governed by the formation of the adduct and its decay. Thus, slowing the supply of arachidonic acid can decrease the ESR signal height even if, after a certain time, the same amount of arachidonic acid was available as if added exogenously.

An initial perplexing problem was the observation that addition of GSH to incubations with cells enhanced thiyl radical formation. In general, GSH is considered not to penetrate cell membranes. Recent data, however, indicate that GSH can be moved from the extracellular to the intracellular (20–21) space, effectively increasing GSH concentration within the cell. We have examined the uptake of GSH by keratinocytes and have found that these cells take up exogenous GSH, yielding a higher intracellular concentration of this tripeptide. This uptake is rapid. Using a membrane vesicle preparation, Vincenzini et al. (27) demonstrated about 1/4 of maximal GSH uptake had occurred after 1 min of exposure to 0.045 mM extracellular GSH. We noted a substantial increase in intracellular GSH levels as early as 3 min after exposure to extracellular GSH. Thus, there was a substantial increase in intracellular GSH in the cells by the time the spectra in Figs. 3 and 4 were obtained. These findings provide a suitable explanation for the observation that the addition of exogenous GSH to cells increases the formation of a thiyl radical.

The formation of the thiyl radical appeared to be intracellular. This conclusion is based primarily upon studies comparing phenol and aminopyrine enhancement of thiyl radical formation and conversion of arachidonic acid to prostaglandins. With ram seminal vesicle and keratinocyte microsomal systems, both peroxidase substrates were equally effective in enhancing thiyl radical and prostaglandin formation, but with the keratinocytes, only phenol significantly enhanced thiyl radical and prostaglandin formation. The basis for the difference between aminopyrine and phenol is not clear. Phenol likely penetrates cell membranes more readily than aminopyrine as the oil/water partition coefficient of phenol is more than 20-fold greater than that of aminopyrine (28), the partition coefficient being the main determinant of a chemical's nonfacilitated cellular permeability (29). Technical problems prevented us from ascertaining uptake. The major difference in the systems compared in Table II is the presence of a cell wall. In the presence of cell walls there was no aminopyrine-enhanced DMPO/GS' thiyl radical adduct signal, whereas in the absence of cell walls, there was a readily detectable signal. Rather than evoke less likely explanations, we think these data support our conclusion that thiyl radical formation occurs inside the keratinocyte.

![Scheme 2. Proposed pathway for phenol co-oxidation in keratinocytes.](image-url)
In summary, we have presented evidence that the formation of a thiyl radical is catalyzed by prostaglandin hydroperoxidase present in mouse keratinocytes. Thiyl radical formation, detected by a spin-trapping technique, was enhanced by the addition of phenol and by elevating intracellular GSH concentration as depicted in Scheme 2. The data indicate that prostaglandin hydroperoxidase-mediated oxidation of chemicals to free radical metabolites can occur in cells.

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