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Endogenous intracellular glutathionyl radicals are generated in neuroblastoma cells under hydrogen peroxide oxidative stress

(free radical/spin trapping/electron paramagnetic resonance/glutathione/*N*-acetyl-L-cysteine)

HAHN-S. KWAK*, HYUNG-S. YIM, P. BOON CHOCK, AND MOON B. YIM†

Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Building 3, Room 202, Bethesda, MD 20892

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ABSTRACT **We report the detection of endogenous intracellular glutathionyl (GS•) radicals in the intact neuroblastoma cell line NCB-20 under oxidative stress. Spin-trapping and electron paramagnetic resonance (EPR) spectroscopic methods were used for monitoring the radicals. The cells incubated with the spin trap 5,5-dimethyl-1-pyrroline 1-oxide (DMPO) were challenged with H_2O_2 generated by the enzymic reaction of glucose/glucose oxidase. These cells exhibit the EPR spectrum of the GS• radical adduct of DMPO (DMPO–•SG) without exogenous reduced glutathione (GSH). The identity of this radical adduct was confirmed by observing hyperfine coupling constants identical to previously reported values in *in vitro* studies, which utilized known enzymic reactions, such as horseradish peroxidase and Cu/Zn superoxide dismutase, with GSH and H_2O_2 as substrates. The formation of the GS• radicals required viable cells and continuous biosynthesis of GSH. No significant effect on the resonance amplitude by the addition of a membrane-impermeable paramagnetic broadening agent indicated that these radicals were located inside the intact cell. *N*-Acetyl-L-cysteine (NAC)-treated cells produced NAC-derived free radicals (NAC•) in place of GS• radicals. The time course studies showed that DMPO–•SG formation exhibited a large increase in its concentration after a lag period, whereas DMPO–NAC• formation from NAC-treated cells did not show this sudden increase. These results were discussed in terms of the limit of antioxidant enzyme defenses in cells and the potential role of the GS• radical burst in activation of the transcription nuclear factor NF-κB in response to oxidative stress.**

Oxidative stress is believed to increase the concentration of reactive oxygen free radicals *in vivo*, which are responsible, at least in part, for a wide variety of degenerative processes (1, 2). Recent studies also demonstrated that treatment of cultured cells with H_2O_2 or other stimuli for oxidative stress leads to the activation of nuclear factor κB (NF-κB) (3–8) and, in turn, induces expression and replication of the human immunodeficiency virus (3–7). These investigations suggest that reactive oxygen intermediates or free radicals serve as messengers for activation of transcription factors. Although pathological and *in vitro* studies lead to the suggestion that toxic free radicals are responsible for oxidative damage, more understanding is required at the cellular level about the mechanisms responsible for the formation of oxygen radicals, their cascading reactions, and cellular signals to genomic levels for activation of defense or repair systems. The fundamental importance in this respect is to find whether free radicals exist in viable cells and whether oxidative stress increases the concentration of the free radicals in viable cells. However, there is a paucity of direct detection and structural identification of free radicals formed in intact cells because of inherent experimental difficulties (9).

In addition to the protection of cells by antioxidant enzymes, low molecular weight antioxidant compounds are thought to be involved in the protection of biomolecules against reactive free radical species, such as hydroxyl radicals (•OH) or other radicals from xenobiotics, which cannot be removed by enzymes. Cellularly abundant reduced glutathione (GSH) is an important molecule in this regard because it functions both as a substrate or cofactor of antioxidant enzymes and as a good radical scavenger (10–14). As a radical scavenger, glutathione repairs free radical sites, R•, by the following reaction:

$$GSH + R^{\bullet} \rightarrow GS^{\bullet} + RH \quad [1]$$

where GS• is the glutathionyl radical and R• represents •OH, other reactive radicals, or radical sites in proteins and DNA generated by cascading reactions (15, 16). When GSH deficiency is produced in newborn rats or guinea pigs, the animals develop multiorgan failure, which is directly related to the loss of essential antioxidant capacity (13, 14, 17, 18). Also, the activation of NF-κB is inhibited by the addition of *N*-acetyl-L-cysteine (NAC), which serves as an oxygen radical scavenger and as a precursor for GSH biosynthesis (4–7). Most of the reaction rates involving GSH in reaction 1 are diffusion controlled (19, 20). Therefore, the glutathionyl radical or its cascading radicals, rather than the initial oxygen free radicals, are likely the predominant intracellular radical species.

We attempted to detect and identify free radicals formed under H_2O_2 stress in intact cells by using electron paramagnetic resonance (EPR) and spin-trapping methods. Our results show that H_2O_2 stress generates endogenous intracellular GS• radicals in intact cells and NAC-originated thiyl radicals (NAC•) in NAC-treated cells. In addition, GS• formation exhibited a large increase in its concentration after a lag period, whereas NAC• formation did not show this sudden increase.

MATERIALS AND METHODS

Materials. The spin trap 5,5-dimethyl-1-pyrroline 1-oxide (DMPO), chromium oxalate, and 2-vinylpyridine were purchased from Aldrich. Horseradish peroxidase (HRP) (type VI), EDTA, GSH, 5,5′-dithiobis(2-nitrobenzoic acid), and cytochrome *c* (type VI, from horse heart) were obtained from Sigma. Glucose oxidase, glutathione peroxidase (bovine erythrocytes), glutathione reductase (yeast), NADPH, oxidized glutathione, deoxycholate, and NAC were purchased from Calbiochem. Cu/Zn superoxide dismutase (Cu/Zn-SOD), xanthine oxidase, and leupeptin were purchased from Boehringer Mannheim; Chelex 100 resin and SDS were from Bio-

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Abbreviations: GSH, reduced glutathione; DMPO, 5,5-dimethyl-1-pyrroline 1-oxide; NAC, *N*-acetyl-L-cysteine; HRP, horseradish peroxidase; SOD, superoxide dismutase; EPR, electron paramagnetic resonance.

*Present address: Department of Genetic Engineering, Pai-Chai University, Taejon, Korea.
†To whom reprint requests should be addressed.

Rad; and H_2O_2 (30%) and picric acid were from Fisher. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and trypan blue were obtained from GIBCO. BCA protein assay reagent was from Pierce, and Triton X-100 was from Research Products International. All solutions were treated with Chelex 100. The pH values of the bicarbonate buffer (23.5 mM) and of bicarbonate-buffered saline (BBS) were adjusted to 7.4 by bubbling 5% CO_2/95% N_2 or air/gas mixture.

Methods. The spin-trapping method utilizes an addition reaction of transient free radicals to diamagnetic spin traps to produce relatively stable spin-trap–free radical adducts (21, 22). The identity of the free radical can be determined from hyperfine coupling constants of the spin adduct by EPR spectroscopy. A spin trap, DMPO, was used in this study. EPR spectra were recorded on a Bruker ESP 300 spectrometer with a TM_{110} resonator. The spectrometer settings were as follows: modulation amplitude, 1.6 G; conversion time, 41 ms; time constant, 0.328 s; resolution of field axis, 2048 points; sweep width, 100 G; sweep time, 84 s; accumulation, two times.

Cell Preparation. NCB-20 cells (kindly provided by M. Nirenberg, National Institutes of Health, Bethesda, MD) were grown in monolayer in DMEM supplemented with 10% (vol/vol) FBS on culture dishes in 5% CO_2/95% air. At confluency, the medium was removed from the dishes and the attached cells were washed twice with Chelex 100-treated BBS at pH 7.4. Incubations were continued in BBS containing 100 mM DMPO for 50 min at 37°C. Viability of cells was determined by the trypan blue exclusion method with cells being removed by gentle scraping from plates. The medium was removed by centrifugation, glucose and glucose oxidase in BBS were added, and cells were incubated again for 30 min at 37°C. The EPR sample (140 μl) contains 1.0×10^7 cells, 7.5 mM glucose, and 200 units of glucose oxidase in a gas-permeable Teflon tube (0.032-inch i.d., 0.002-inch wall; Zeus Industrial Products, Orangeburg, SC). The H_2O_2 produced under these conditions is $\approx$300 μM. The sample tube was folded (four turns) and inserted into a quartz EPR tube open at each end and positioned in the horizontally mounted TM_{110} resonator. The quartz tube was connected to a 5% CO_2/95% air cylinder. Spectra were recorded at 25°C. No GSH was supplemented in these samples.

For determination of antioxidant enzyme activities, confluent cells were rinsed twice with PBS, collected, and sedimented for 5 min at room temperature at 800 rpm. Cells were resuspended in lysis buffer (50 mM potassium phosphate buffer containing 0.2% Triton X-100 and 0.1 mM EDTA) for 1 hr and centrifuged for 20 min at 14,000 rpm (16,000 $\times g$). The total protein concentration was determined according to the procedure of the manufacturer (Pierce) with BCA (bicinchoninic acid based) protein assay reagent. The protein concentration of one EPR sample was $\approx$3 mg. The enzyme activities were assayed according to the procedures of Clairborne (23) for catalase, Gunzler and Flohé (24) for glutathione peroxidase, and McCord and Fridovich (25) for Cu/Zn-SOD (cyanide-sensitive activity) and Mn-SOD (cyanide-insensitive activity). Glutathione concentration was measured by the procedure reviewed by Anderson (26).

RESULTS

Observation of Glutathionyl Radicals in NCB-20 Cells. The EPR spectrum A in Fig. 1 was obtained when NCB-20 cells incubated in BBS containing 100 mM DMPO were subjected to oxidative stress exerted by H_2O_2 for 100 min. H_2O_2 (300 μM) required for this observation was generated extracellularly by an enzymic reaction of the glucose (7.5 mM) and glucose oxidase (200 units) systems. Hyperfine coupling constants obtained from this spectrum (a^N = 15.30 G and a^H = 16.35 G) were characteristic for DMPO–$^{\bullet}$SG, the glutathionyl radical adduct of DMPO (21, 27–32). To confirm the identity of the radical species, GS$^{\bullet}$ radicals were generated in solution by known enzymic reactions. Previous studies (21, 29) showed that DMPO can trap GS$^{\bullet}$ radicals generated by the enzymic reaction of HRP in the presence of GSH and H_2O_2. Spectrum B shown in Fig. 1 was obtained with a similar reaction and it is identical to spectrum A that was obtained with NCB-20 cells.

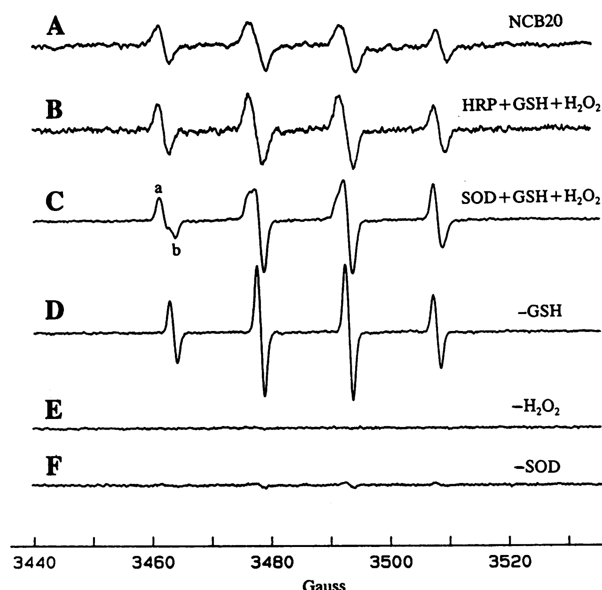


FIG. 1. EPR spectra of DMPO–free radical adducts observed in NCB-20 cells and in known enzymic reactions. Spectrum A, the EPR sample from NCB-20 cells was recorded 100 min after addition of glucose oxidase at 25°C under the conditions described in *Materials and Methods.* Spectrum B, the reaction mixture under anaerobic conditions contained 0.1 mg of HRP per ml, 0.05 mM H_2O_2, 10 mM GSH, and 60 mM DMPO in 20 mM Hepes buffer at pH 7.2. The spectrum was taken with the sample in the quartz flat cell positioned in a vertically mounted TM_{110} resonator 1 min after the reaction was initiated by injecting H_2O_2. The spectrometer settings were identical to those in spectrum A except conversion time was 20.48 ms and resolution was 4096 points. Spectrum C, the reaction mixture contained 0.1 mg of Cu/Zn-SOD per ml, 2.0 mM H_2O_2, 20 mM GSH, and 10 mM DMPO in 23.5 mM bicarbonate buffer at pH 7.6. Other conditions are identical to those in spectrum B. The experimental conditions for spectra D, E, and F were identical to those for spectrum C except that GSH, H_2O_2, and Cu/Zn-SOD were omitted for spectra D–F, respectively.

We have shown previously (33, 34) that Cu/Zn-SOD can catalyze the formation of $^{\bullet}$OH radicals with H_2O_2 as substrate and the formation of scavenger radicals with anionic radical scavengers and H_2O_2 as the substrates. On the basis of these findings, GS$^{\bullet}$ radicals were also generated by the catalytic reaction of Cu/Zn-SOD in the presence of GSH and H_2O_2 (Fig. 1, spectrum C). The EPR signal pattern of spectrum C indicates the presence of at least two radical adduct species (marked a and b) generated by this reaction. In the absence of GSH, a four-line EPR signal of DMPO–$^{\bullet}$OH adducts ($a^N = a^H$ = 14.9 G) was detected (Fig. 1, spectrum D). The position of the low-field line is identical to that of b in spectrum C. When spectrum D was subtracted from spectrum C after normalization, an EPR spectrum identical to A or B was obtained. As shown in spectra E and F (Fig. 1), the formation of $^{\bullet}$OH radicals requires both the enzyme and H_2O_2. In addition, GSH is also required for formation of DMPO–$^{\bullet}$SG radical adducts. However, in experiments with NCB-20 cells, exogenous glutathione was not necessary for observation of DMPO–$^{\bullet}$SG radical adducts (spectrum A). It indicates that GSH is continuously synthesized intracellularly and is a primary radical scavenger in cells during oxidative stress exerted by H_2O_2.

After observing the DMPO–$^{\bullet}$SG signal, the cells were removed from the sample tube and counted with the addition of trypan blue. The result indicates that >95% of cells were in an intact state after 3 hr of exposure to H_2O_2.

Intracellular Production of Glutathionyl Radicals. It is important to determine whether the GS$^{\bullet}$ radicals observed were generated in the intracellular or extracellular space. Spectrum A in Fig. 2 is identical to spectrum A in Fig. 1 detected at 25°C. When the sample temperature was lowered to 12°C, the signal intensity of DMPO–$^{\bullet}$SG radical adducts was greatly reduced (Fig. 2, spectrum B). However, the resonance amplitude was restored when the sample temperature was raised to 25°C again. The omission of glucose oxidase resulted in no detectable radical adducts (Fig. 2, spectrum C), indicating that a source of H_2O_2 diffusible across the cell wall is required for the detectable free radical signal. These observations may suggest that the biosynthesis of glutathione and/or metabolism involving H_2O_2 for initiating a free radical reaction should be maintained as a physiological condition in intact cells.

To further investigate the space where DMPO–$^{\bullet}$SG adducts were formed, a similar experiment was performed in the presence of chromium oxalate, a charged, membrane-impermeable paramagnetic broadening agent (9, 35–37). The chromium oxalate remains exclusively in the extracellular space of intact cells and broadens away the resonance signal from extracellular radical adducts. Under these conditions, the observed signal can be attributed only to the intracellular radical adducts. After obtaining spectrum A in Fig. 2, the

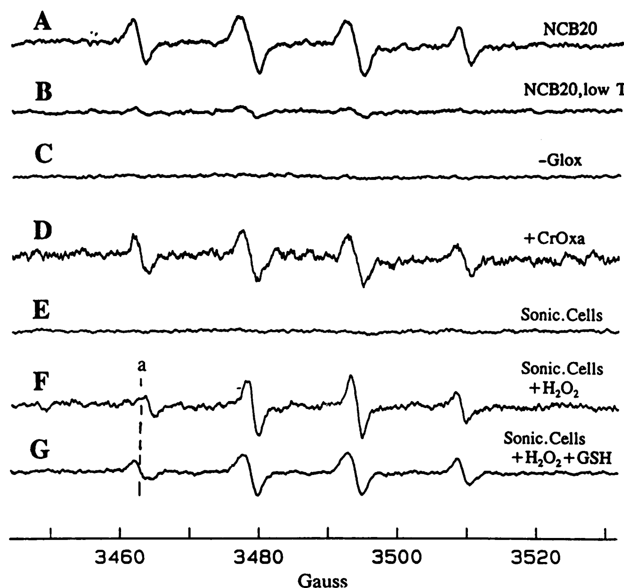


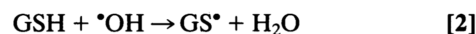
FIG. 2. Requirement of viable NCB-20 cells for formation of glutathionyl radicals. Spectrum A, this spectrum is identical to spectrum A in Fig. 1. Spectrum B, after recording spectrum A, the samples were cooled to 12°C and the spectrum was retaken. Spectrum C, all conditions were identical to those in spectrum A except that the sample did not contain glucose oxidase (Glox). Spectrum D, the chromium oxalate (CrOxa) (10 mM) was added to the sample used in spectrum A. All other conditions were identical to spectrum A except that the receiver gain for the spectrometer was 2-fold to compensate for the 2-fold dilution of the sample. Spectrum E, the cells in a solution similar to that in spectrum A were disrupted by sonication and the spectrum was retaken. All other conditions were identical to those for spectrum A. Spectrum F, the sample was the cytosolic portion of disrupted cell extracts similar to that in spectrum E except that DMPO (60 mM) and H_2O_2 (1 mM) in place of glucose and glucose oxidase were added. The spectrum was taken with a quartz flat cell in a vertically mounted TM_{110} resonator. Spectrum G, the sample was similar to that in spectrum F except that 10 mM GSH was also added.

sample was removed from the Teflon tube and an equal volume of chromium oxalate solution (10 mM) in BBS was added. Spectrum D was obtained from this sample with instrumental conditions identical to those for spectrum A except that the receiver gain was increased by a factor of 2. The signal amplitude of DMPO–$^{\bullet}$SG radical adducts shown in spectrum D is unchanged relative to that of spectrum A. When DMPO–$^{\bullet}$SG adducts were generated in solution without cells, addition of chromium oxalate completely broadened the EPR signal beyond detection (data not shown). These results indicate that DMPO–$^{\bullet}$SG adducts were formed in the intracellular space of intact cells.

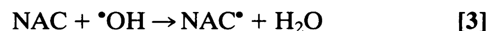
To demonstrate that viable cells are required for the observation of DMPO–$^{\bullet}$SG radical adducts, a sample identical to that used for obtaining spectrum A was subjected to brief sonication for cell disruption. This treatment abolished the resonance signal (Fig. 2, spectrum E). The addition of DMPO (60 mM) and H_2O_2 (1 mM) in place of glucose/glucose oxidase to the cytosolic portion of the ruptured cell extracts resulted in spectrum F. The resonance lines in this spectrum are mainly from DMPO–$^{\bullet}$OH radical adducts with a very minor contribution from DMPO–$^{\bullet}$SG radical adducts (marked by a), which disappeared rapidly. However, the addition of GSH (10 mM) together with H_2O_2 restored EPR signals for DMPO–$^{\bullet}$SG radical adducts (Fig. 2, spectrum G). These results together indicate that, although initiation of free radical reactions (probably by generating $^{\bullet}$OH radicals from H_2O_2) can occur in the cytosolic portion of cell extracts, intact cells are required for formation of GS$^{\bullet}$ radicals. These radicals were derived from scavenging initial radicals by the endogenous GSH in cells. The GSH must be continuously biosynthesized in cells to support this reaction.

The quantity of antioxidant enzymes in the soluble fraction of NCB-20 cell extracts was determined. The levels of antioxidant enzymes seem to be normal in this cell: catalase, 12.2 units per mg of protein; glutathione peroxidase, 80.5 units/mg; Cu/Zn-SOD, 13.6 units/mg; Mn-SOD, 6.0 units/mg. The observation of GS$^{\bullet}$ radicals in NCB-20 cells was not, therefore, caused by any missing antioxidant enzyme.

Effect of NAC on Formation of Free Radicals. NAC that enters cells readily has been suggested to serve both as a scavenger for reactive radical species and as a precursor for GSH by raising the intracellular concentration of cysteine (4, 14, 31). To study the effect of NAC, NCB-20 cells were incubated with NAC (10 mM) in BBS medium for 60 min prior to incubation with DMPO and with glucose/glucose oxidase. Fig. 3 shows that the presence of NAC caused an alteration of the EPR spectrum (spectrum B) relative to the control (spectrum A). Hyperfine coupling constants obtained from simulation (lower spectrum of B: $a^H = 16.80$ G, $a^N = 15.17$ G) are identical to the reported values of DMPO–NAC$^{\bullet}$ radical adducts (31). Also, the close match between the experimental and the simulated spectra indicates that a negligible concentration of GS$^{\bullet}$ radicals was produced under these conditions. The hydroxyl radical scavenging ability of GSH is approximately equal to that of NAC based on the rate constants determined for the reactions shown below:



$$\mathrm{GSH} + {}^{\bullet}\mathrm{OH} \rightarrow \mathrm{GS}^{\bullet} + \mathrm{H_2O} \qquad [2]$$



$$\mathrm{NAC} + {}^{\bullet}\mathrm{OH} \rightarrow \mathrm{NAC}^{\bullet} + \mathrm{H_2O} \qquad [3]$$

The reported rate constants are $k_2 = 1.5 \times 10^{10}\ \mathrm{M^{-1} \cdot s^{-1}}$ (19) and $k_3 = 1.36 \times 10^{10}\ \mathrm{M^{-1} \cdot s^{-1}}$ (20) for reactions **2** and **3**, respectively. The observation of a negligible amount of DMPO–$^{\bullet}$SG radical adducts in this experiment indicates that NAC-treated cells contain a much higher concentration of NAC than GSH.

Time Course of Thiyl Radical Formation in NCB-20 Cells. Fig. 4 shows the time courses of resonance amplitudes of

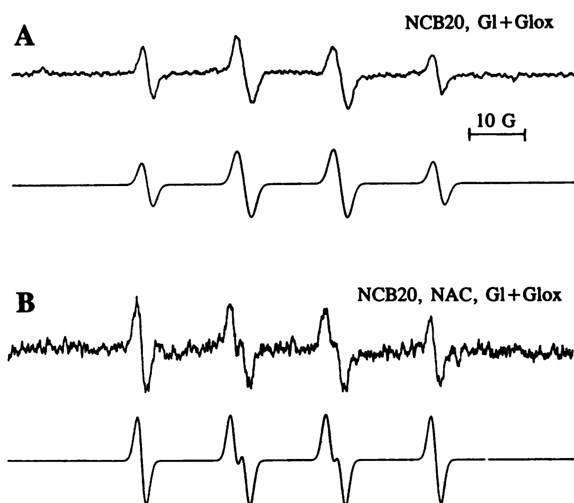


FIG. 3. Thiyl radicals observed in viable NCB-20 cells treated with NAC. Spectra A, the EPR spectrum of DMPO–•SG radical adducts (*Upper*) is identical to spectrum A in Fig. 1. The simulation of the spectrum (*Lower*) was performed by using the following parameters: hyperfine coupling constants, a^H = 16.35 G and a^N = 15.30 G; second-order calculation and Gaussian lineshape with 2.2-G linewidth. Spectra B, the experimental spectrum (*Upper*) was obtained with a sample prepared similarly to that in spectra A except that cells were incubated for 60 min in the medium containing 10 mM NAC prior to the addition of DMPO. The spectrometer settings were as follows: modulation amplitude, 0.8 G; conversion time, 82 ms; time constant, 0.328 s; resolution, 2048 points; sweep width, 100 G; sweep time, 167.8 s. The simulated spectrum (*Lower*) was obtained by using the following parameters: hyperfine coupling constants, a^H = 16.80 G and a^N = 15.17 G; second-order calculation and Gaussian lineshape with 1.8-G linewidth. Gl, glucose; Glox, glucose oxidase.

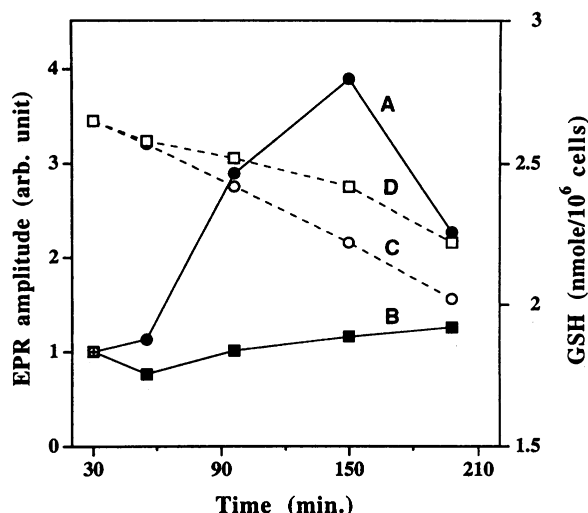


FIG. 4. Time course of thiyl radical formation and glutathione concentration in NCB-20 cells during H_2O_2 stress. The ordinate for curves A and B is on the left, and that for curves C and D is on the right. Curve A, the amplitudes of the first line at low magnetic field of DMPO–•SG spectra as shown in Fig. 1, spectrum A, were obtained at the indicated time and compared with that obtained 30 min after addition of glucose/glucose oxidase. The cell preparation for EPR samples and spectrometer setting are identical to those described in Fig. 1, spectrum A. After each measurement, the sample tube was removed from the resonator and incubated in a cell culture incubator for the next measurement. Curve B, the first line at low field of DMPO–NAC• spectra was obtained at the indicated time and compared with those of DMPO–•SG shown in curve A. Experimental conditions and spectrometer settings are identical to curve A except that NAC (10 mM) was incubated for 60 min before the addition of DMPO. Curve C, change in GSH concentration in NCB-20 cells. Curve D, change in GSH concentration in NAC-treated cells.

DMPO–•SG (curve A) and DMPO–NAC• (curve B) adducts observed after the addition of glucose oxidase to generate H_2O_2. Without addition of NAC, the formation of endogenous DMPO–•SG proceeded through an induction period for ≈60 min and reached 4-fold at 2.5 hr. However, the intracellular concentration of GSH, which is the GS• radical precursor, decreased during this period (curve C). These results indicate that the antioxidant defense system in cells is well regulated during the induction period and effectively removes H_2O_2 and/or GS• radicals, utilizing the catalytic activity of catalase, peroxidase, and thiol-specific antioxidant enzyme systems (21, 38, 39).

When NCB-20 cells were incubated with NAC for 60 min, the concentration of DMPO–thiyl radical adducts remained low and almost constant for 3.5 hr (Fig. 4, curve B). The thiyl radical adducts observed during this period were identified to be DMPO–NAC•. However, the intracellular concentration of GSH (Fig. 4, curve D) decreased during this period, as was observed in the absence of NAC. If NAC functions solely as a radical scavenger that competes for the initial radicals with GSH according to reactions **2** and **3**, the time course shown by curve B should exhibit behavior similar to that depicted in curve A for DMPO–•SG formation. These differences, both in signal intensity and in the kinetic patterns observed for DMPO–NAC• and DMPO–•SG adducts, suggest that NAC functions differently in response to oxidative stress. NAC appears to have two roles—on one hand, it functions as a radical scavenger to form NAC• radicals as in the case of GSH and on the other hand, through a yet to be identified mechanism, it maintains a low steady-state level of free radicals in cells.

DISCUSSION

To detect intracellular free radicals, one has to overcome the cellular metabolism of oxidants by antioxidant enzymes as well as the low steady-state concentration of cascading free radicals. An investigation has been reported by Schreiber *et al.* (32) for trapping GS• in keratinocytes produced by the addition of exogenous phenol, arachidonic acid, GSH, and DMPO.

In our experiment, NCB-20 cells were continuously challenged with extracellularly produced H_2O_2 without any addition other than DMPO. This approach allows one to reveal the ability of intracellular antioxidant enzyme defenses as well as the identity of free radicals formed at their defensive limit. Our results indicate that GS• radicals were produced at this limit. The observation of the radical adduct signal required both intact cells (Fig. 2, spectrum E; sonication effect) and the continuous biosynthesis of glutathione (Fig. 2, spectra B and E–G; temperature and cell disruption effects). In addition, these radicals were found to be located in the intracellular space on the basis of the broadening experiment with chromium oxalate (Fig. 2, spectrum D), which is an essential experiment to demonstrate the intracellular nature of radicals. The identity of the trapped radicals was further confirmed as GS• radicals by showing that Fig. 1 (spectrum A) is identical to EPR spectra of authentic DMPO–•SG generated by known enzymic reactions of HRP (21, 29, 31) or Cu/Zn-SOD (33, 34) in the presence of both GSH and H_2O_2 (Fig. 1, spectra B and C).

The time course of endogenous intracellular DMPO–•SG adduct formation exhibited a large increase in its concentration after a lag period (Fig. 4, curve A). However, the concentration of endogenous glutathione, the precursor for the DMPO–•SG radical adduct, decreased continuously during H_2O_2 exposure (Fig. 4, curve C). These results suggest that the protective mechanism of antioxidant enzymes against oxidative stress in cells became inefficient after the lag period under our experimental conditions. The effect of NAC on the formation of DMPO–thiyl radical adducts (Fig. 4, curve B) is

surprising. If NAC behaves solely as a direct radical scavenger to compete with endogenous GSH for initial radicals and as a substrate for glutathione biosynthesis as generally thought, the concentration of DMPO–NAC• radical adducts must be as high as that of DMPO–•SG observed in the absence of NAC. On the contrary, the time course of the total DMPO–thiyl radical adduct formation shows a different kinetic pattern in which NAC maintains a low concentration of radical adducts (thus the lag period) for >3 hr. This result therefore indicates that NAC may also protect efficient regulation of antioxidant enzyme systems against inactivation in addition to its roles as a direct radical scavenger and a substrate for glutathione biosynthesis.

Recent studies demonstrated that treatment of cells with H_2O_2 or other oxidative stress agents activates the NF-κB from its inactive cytoplasmic form by releasing the inhibitory subunit IκB from NF-κB and in turn promoting expression of genes controlled by the long terminal repeat of the human immunodeficiency virus (3–6). It was also reported that NAC blocks this NF-κB activation (3–7). These investigations suggested that reactive oxygen intermediates serve as messengers mediating directly or indirectly the activation of NF-κB and also that intracellular thiol levels play a key role in regulating this process. Meyer *et al.* (7) also showed that H_2O_2 (150 μM) treatment of HeLa cells transactivated NF-κB with an ≈60-min lag period and NAC (30 mM) inhibited this transactivation. Their results are surprisingly similar to our time courses shown in Fig. 4 for the formation of GS• radicals and the effect of NAC when the cells were exposed to H_2O_2. With this correlation, it is tempting to suggest that the sudden increase of GS• radical formation after a lag period in intact cells may be responsible for or indicative of the activation of NF-κB. The treatment of NAC, which lowered the steady-state concentration of thiyl radicals by a yet to be identified mechanism, also inhibited the activation of NF-κB. Thus, our findings may have significance for understanding how oxidative stress mediates the activation of the NF-κB.

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