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OXYGEN RADICAL FORMATION IN WELL-WASHED RAT LIVER MICROSOMES: SPIN TRAPPING STUDIES

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The generation of hydroxyl radicals by rat liver microsomes was monitored by spin trapping with 5,5-dimethylpyrroline N-oxide (DMPO). The results confirm and extend previous data which demonstrated that hydroxyl radicals are produced by microsomes in the presence of NADPH and O_2, and without the exogenous addition of iron. No EPR signals could be detected unless catalase activity which was associated with the microsomes could be substantially diminished. Addition of azide was the most effective means of eliminating catalase activity, but azide also reacted rapidly with hydroxyl radicals, forming azidyl radicals which were in turn trapped by DMPO. Extensive washing and preincubation of microsomes with 3-amino-1,2,4-triazole in the presence of H_2O_2 were evaluated as alternative methods of decreasing the catalase contamination of microsomes. Although neither method completely eliminated microsomal catalase activity, addition of azide was no longer necessary for hydroxyl radical detection with DMPO. When highly washed microsomal preparations were tested, weak signals of the superoxide radical adduct of DMPO could also be detected. These data indicate that the sensitivity of spin trapping in microsomal systems can be improved substantially when care is taken to eliminate cytosolic contaminants such as catalase.

KEY WORDS: Catalase, DMPO, hydroxyl radicals, liver microsomes, spin trapping, superoxide.

INTRODUCTION

Hepatic microsomes catalyze a wide variety of biological oxidations which require molecular oxygen and NADPH. Many of these reactions are catalyzed by the cytochrome P-450 family of isozymes, in which the substrate is oxidized while bound at the catalytic site of the hemoprotein.[1,2] Other microsomal oxidations may occur through formation of a hydroxyl radical (·OH) intermediate. Examples of these reactions include oxidation of alcohols,[3-7] dimethylsulfoxide[6] and aromatic compounds;[7,8] decarboxylation of benzoate;[9] and initiation of lipid peroxidation.[10] In the mechanism usually proposed for these reactions, H_2O_2 formed by microsomes in the presence of O_2 and NADPH reacts with reduced iron to generate

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·OH in a Fenton or iron-catalyzed Haber-Weiss reaction. Because of the high reactivity of ·OH, this intermediate cannot be detected directly, and evidence supporting its involvement in a given reaction often consists of inhibitory effects of catalase, iron-chelating compounds, or oxygen radical scavengers.[3-9] Unfortunately, some scavengers may compete for cytochrome P-450 binding sites and competitively inhibit substrate oxidation, or they may affect the rate of H_2O_2 formation, so that differentiation between cytochrome P-450-catalyzed and ·OH-mediated oxidative mechanisms is difficult. Even if a microsomal oxidation can be shown to be dependent on H_2O_2 and iron, this does not prove a role for ·OH because some other highly reactive iron-oxygen intermediate (e.g. ferryl, perferryl) could be involved.

The spin trapping method offers the possibility of unambiguous identification of free radical intermediates. In spin trapping, a highly reactive radical reacts with a suitable "spin trap" to form a more persistent secondary radical (a "spin adduct") which may be detected and identified by electron paramagnetic resonance (EPR) spectroscopy. 5,5-Dimethylpyrroline N-oxide (DMPO) has been particularly useful in spin trapping studies involving the hydroxyl radical, because relatively stable spin adducts (DMPO-OH) are formed.[11] However, attempts to use spin trapping to directly study microsomal hydroxyl radical formation have typically yielded negative results, unless iron or another appropriate transition metal were also added.[10,12,13]

The failure to detect ·OH in microsomal incubations with spin trapping could indicate that some other intermediate which does not form spin adducts is responsible for reactions which have been attributed to the hydroxyl radical. We have utilized spin trapping to probe possible roles of ·OH in the metabolism of ethanol to the 1-hydroxyethyl radical in liver microsomes,[14,15] and have shown that catalase which contaminates the microsomes complicates detection of ·OH with DMPO.[15] The data of this report demonstrate that careful preparation of microsomes to remove cytosolic components such as catalase further optimizes spin trapping conditions, so that both hydroxyl and superoxide adducts of DMPO can be observed in the absence of catalase inhibitors.

MATERIALS AND METHODS

Male, Sprague-Dawley rats (Sasco, Omaha NE) were anesthetized with an i.p. injection of sodium pentobarbital, and the livers were perfused through the portal vein with approximately 50 ml of ice-cold saline in order to remove blood from the liver. Unless otherwise indicated, the livers were homogenized in 10 volumes of 0.04 M phosphate buffer, pH 7.4. The homogenates were centrifuged at 10,000 X g at 4°C, and the supernatants were drawn off with a pipet, taking care not to disturb the peroxisomal layer at the top of the pellet. The supernatants were centrifuged at 100,000 X g at 4°C for 60 min to sediment the microsomes. Each wash consisted of resuspending the pellet in a volume of 0.16 M KCl that was equal to that of the 100,000 X g supernatant, and performing another 100,000 X g centrifugation for 30 min. After the final wash, the microsomal pellets were layered with approximately 3 ml of 0.16 M KCl, and were stored at −70°C until used.

In one experiment, microsomes were treated with 3-amino-1,2,4-triazole (AT) in an attempt to inhibit catalase activity.[16] The microsomes were incubated at 37°C for 60 min under an atmosphere of N_2 with no additions, or with H_2O_2 (10 mM) plus AT (10 mM). The microsomes were subsequently washed twice to remove

residual H_2O_2 and AT. Control microsomes from the same initial suspension were incubated under N_2 with no additions, and were also washed twice.

Spin trapping experiments were conducted as described previously.[15] Briefly, microsomes were incubated with DMPO (40 mM), phosphate buffer (40 mM, pH 7.4), and an NADPH-generating system consisting of $NADP^+$ (0.3 mM), glucose-6-phosphate (5 mM), and glucose-6-phosphate dehydrogenase (0.5 units/ml). The protein concentration of the microsomal suspensions ranged from 3 to 5 mg/ml, but the same concentration was used for data shown in each figure so that spectral intensities can be directly compared. Potassium azide and other additions were at concentrations indicated in the text and figure legends. The microsomal incubation systems were assembled and transferred to quartz flat EPR cells, and the reactions were monitored at room temperature in the cavity of a Bruker E-300 spectrophotometer. Typical instrumental settings were as follows: modulation frequency, 100 kHz; modulation amplitude, 0.975 G; conversion time, 328 msec; sweep time, 84 sec; center field, 3480 G; sweep width, 100 G; and microwave frequency, 9.75 GHz.

Other microsomal activities were determined at 37°C by methods established in the literature. Superoxide formation was measured by monitoring the superoxide dismutase-inhibitable reduction of succinylated cytochrome c.[17] Microsomal H_2O_2 production in the presence of azide (1 mM) was quantitated enzymatically using glutathione reductase and glutathione peroxidase.[18] NADPH-cytochrome c reductase activity was measured as a microsomal "marker" as described by Vermillion and Coon.[19] Catalase activity was assayed by disappearance of H_2O_2 at 240 nm,[20] and bathophenanthrolene was used to form a colored complex with ferrous ions in non-heme iron assay.[21] Protein concentrations were measured by the method of Lowry, *et al.*[22]

DMPO was purchased from Sigma, and was purified prior to EPR studies by addition of activated charcoal (approximately 50 mg/ml), and filtration through 0.2 micron Gelman Acrodiscs. Phosphate buffers and KCl solutions used in these studies were prepared in distilled, deionized water, and were thoroughly treated with Chelex 100 resin (Bio-Rad Laboratories). The solutions were subsequently tested for trace metal contamination by monitoring the rate of ascorbate autoxidation.[23] The solutions were only used if the loss of ascorbate absorbance was less than 0.5% in 20 min, indicating effective removal of Fe or Cu by the chelation procedure.[23] After chelation, the pH was adjusted to 7.4 if needed. Biochemicals used in these studies were also purchased from Sigma, and all chemicals used were of high reagent grade purity. Units of superoxide dismutase activity are as defined by McCord and Fridovich,[24] and one unit of catalase degrades 1.0 μmole of H_2O_2 per minute at pH 7.0 and 25°C.

RESULTS

The data shown in Figure 1 indicate typical results obtained when livers were homogenized in three volumes of phosphate buffer, and were washed only once. When microsomes were incubated with DMPO and an NADPH-generating system in an air atmosphere, no EPR signals were observed. However, when azide (0.1 mM) was also added, a weak EPR signal with characteristics of the hydroxyl radical adduct of DMPO (DMPO-OH) was detected (Figure 1), as reported previously.[15] As more azide was added, the EPR signal of the DMPO-OH adduct became more intense, but the signals of a second radical adduct began to appear as well. This

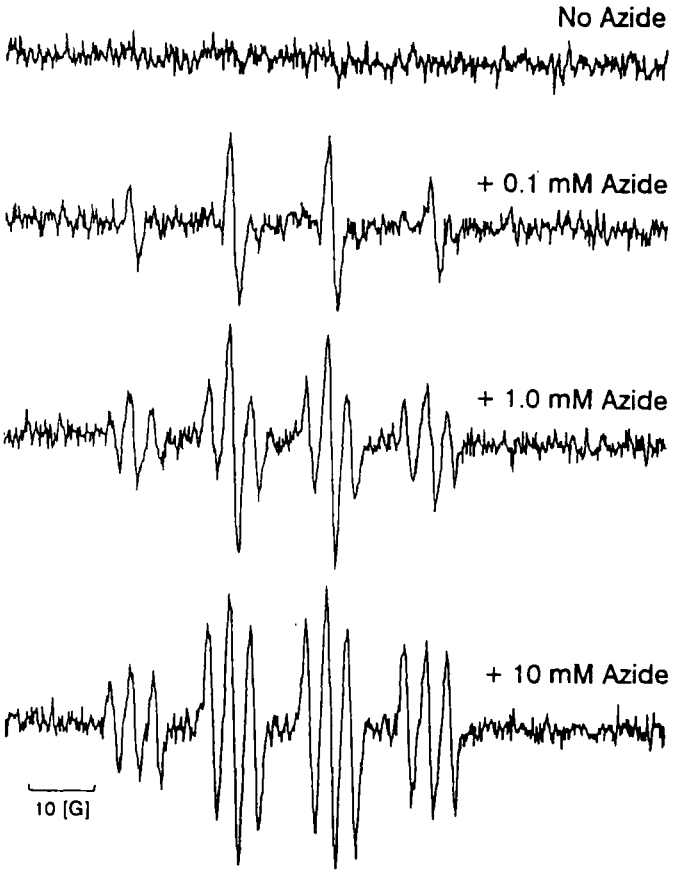

FIGURE 1 *Effect of azide addition on spin trapping with DMPO in rat liver microsomes* Rat liver microsomes were isolated by differential centrifugation, and were washed once. The microsomes were mixed with DMPO (40 mM), an NADPH-generating system, and various concentrations of azide, and were subjected to EPR analysis as indicated in *Materials and Methods*. The spectra shown are from a single scan, using a spectrometer gain of 10^6 and microwave power of 20 mW. The bar represents 10 gauss.

second spin adduct had hyperfine splitting constants that are typical of the azidyl radical adduct of DMPO (DMPO-AZ). When the azide concentration was increased to 10 mM, the DMPO-AZ signal dominated the EPR spectrum (Figure 1).

Azide is a potent inhibitor of catalase, with values for K_i in the range of 10^{-8} to 10^{-6} M.[25] Therefore, the results shown in Figure 1 indicate that catalase which contaminates microsomes might interfere with formation of ·OH by destroying H_2O_2. However, complete inhibition of the contaminating catalase activity required an azide concentration of at least 1 mM (data not shown), and at this concentration the DMPO-Az signal was readily apparent (Figure 1). It was therefore desirable to find other methods of minimizing the catalase contamination of microsomes.

Cytosolic proteins can be effectively removed by washing the microsomes with

TABLE I
Effect of repeated washing on microsomal protein, non-heme iron content, and enzyme activities

Parameter measured	Number of Washes			
	None	One	Two	Three
Protein (mg/ml suspension)	3.68	2.95	2.82	1.92
Catalase (units/mg protein)	104 ± 31	13 ± 5	6 ± 1	8 ± 3
Non-heme iron (nmol/mg protein)	17.5 ± 0.7	10.3 ± 5.6	6.9 ± 3.9	8.2 ± 5.6
Superoxide formation (nmol/min/mg protein)	2.4 ± 0.4	3.4 ± 0.6	3.3 ± 0.9	3.9 ± 0.6
H_2O_2 formation (nmol/min/mg/protein)	1.1 ± 0.7	1.9 ± 0.8	2.2 ± 0.78	2.5 ± 0.8
NADPH-Cytochrome c Reductase (units/mg protein)	82 ± 26	120 ± 13	127 ± 28	164 ± 27
DMPO-OH EPR signal (mm peak ht/mg protein)	9	19	30	43

Livers were homogenized in 10 volumes of phosphate buffer, and the 10,000 X g supernatants were pooled to make a homogeneous suspension. This suspension was divided into a number of equal aliquots from which microsomes were prepared. The suspensions were centrifuged at 100,000 X g to obtain the microsomes, which were used with no further washes, or after one, two, or three washes as described in Methods. Pellets used in these experiments were all resuspended in the same volume of 0.16 M KCl. The protein values are from one experiment of the three that were performed. All other values are means ± SD from at least three measurements that were performed on different days. For catalase, units are defined as nmol of H_2O_2 catabolized/min at room temperature. For cytochrome c reductase, units are defined as nmol cytochrome c reduced/min at 37°C. The EPR signal intensity in the presence of 0.1 mM azide was measured under standard conditions of gain and scale as indicated in Figure 1, and the peak height in mm was standardized by the concentration of protein in the sample.

salts such as KCl,[26] and this approach was evaluated for spin trapping experiments. Livers from 4 rats were homogenized in ten volumes of phosphate buffer in order to dilute cytosolic components which might sediment with the microsomes during isolation. The 10,000 X g supernatants were pooled to make a homogeneous suspension, and equal volumes of the suspension were placed into ultracentrifuge tubes for preparation of microsomes. Various microsomal enzyme activities were measured after the initial 100,000 X g sedimentation, or after one to three washes. Each wash resulted in the loss of total protein in the pellets (Table 1). Concentrations of catalase and non-heme iron, expressed per mg of protein, were decreased by the first two washes, but were not lowered further by a third wash. NADPH-cytochrome c reductase activity increased with each wash, indicating removal of proteins which are not bound to the endoplasmic reticulum (Table 1). Similar increases in rates of microsomal superoxide and H_2O_2 formation were also observed. When spin trapping experiments were conducted to detect the DMPO-OH adduct, the signal intensity also increased with the number of washes (Table 1). Azide (0.1 mM) was used in these EPR experiments to inhibit residual catalase activity, which varied among the various microsomal preparations.

When microsomes which had been washed three times were tested in spin trapping experiments, a weak DMPO-OH signal could be detected in the absence of azide (Figure 2). Addition of azide (0.1 mM) intensified the EPR signal, which could reasonably be attributed to inhibition of residual catalase activity (Table 1). However, it is also possible that azide directly participates in the formation

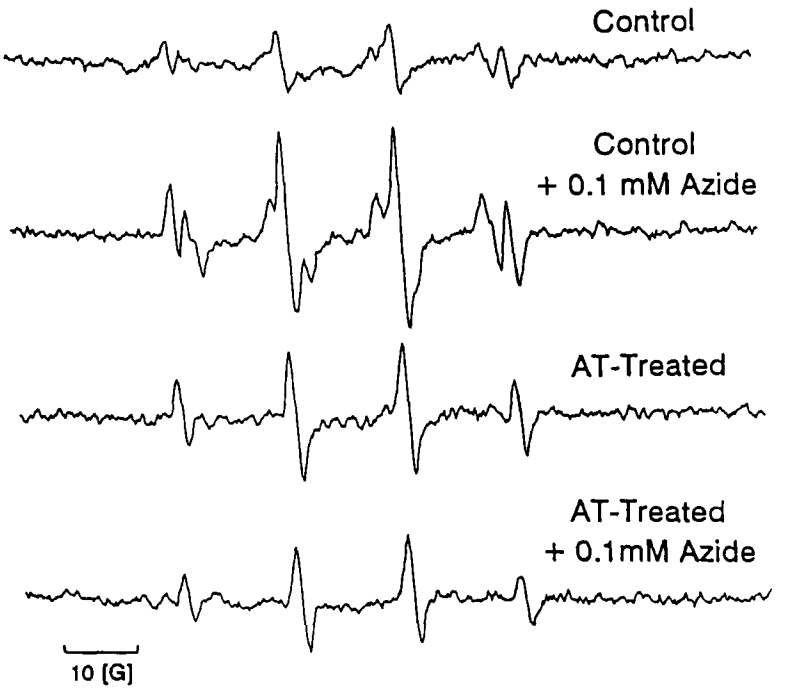

FIGURE 2 *EPR spectra from extensively washed microsomes*
Microsomes were washed three times in 0.16 M KCl, and spin trapping experiments were conducted in the absence or presence of 0.1 mM azide. Some microsomes from the same initial suspension were also incubated with 3-amino-1,2,4-triazole (AT, 10 mM) and H_2O_2 (10 mM) as described in Materials and Methods, and were otherwise treated as the control microsomes. Spectrometer conditions were as indicated in the legend of Figure 1, except that 10 scans of 84 sec were accumulated.

of DMPO-OH spin adducts. This problem was investigated through the use of 3-amino-1,2,4-triazole (AT), which is converted into an irreversible inhibitor of catalase in the presence of H_2O_2.[16] Microsomal suspensions were incubated *in vitro* with AT and H_2O_2 as indicated in Materials and Methods. AT treatment was relatively ineffective in blocking catalase activity (6.5 ± 0.5 and 4.5 ● 0.6 units/mg microsomal protein in control and AT-treated microsomes, respectively). However, the DMPO-OH signal intensity in AT-treated microsomes was about twice that of control microsomes in the absence of azide, and was not increased by addition of azide (Figure 2). In these spectra obtained from highly washed microsomes, there was evidence of a second spin adduct with a weak EPR signal (Figure 2).

The effects of catalase or superoxide dismutase (SOD) on spin trapping with DMPO were tested with microsomes that had been isolated after three washes. In the absence of added enzymes or azide, the predominant signal was that of the DMPO-OH adduct (Figure 3). Addition of SOD had little effect on the signal intensity of the DMPO-OH adduct, but it eliminated the weak EPR signals observed in the absence of the enzyme (Figure 3). These results with SOD indicate that the weak EPR signals are due to the superoxide radical adduct of DMPO (DMPO-OOH). Catalase addition eliminated the DMPO-OH signal, and left a weak EPR signal with characteristics of the DMPO-OOH spectrum. Addition of both enzymes resulted in the total loss of EPR signals (Figure 3).

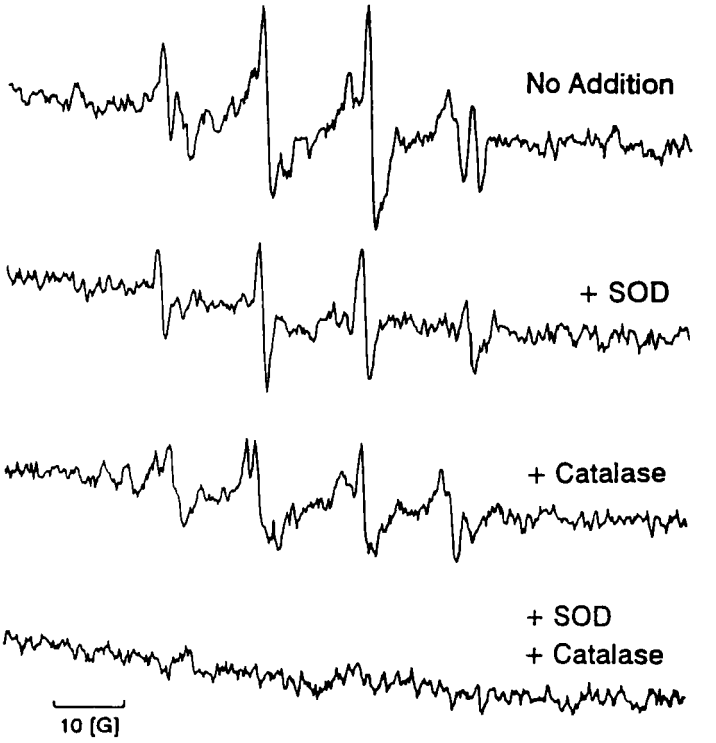

FIGURE 3 *Effects of superoxide dismutase and catalase on spin trapping of oxygen radicals in rat liver microsomes*
Spin trapping experiments were conducted with microsomes that had been washed three times, and were run in the absence or presence of superoxide dismutase (100 units/ml) or catalase (100 units/ml). Other conditions are as indicated in the legend of Figure 2.

DISCUSSION

The results of these experiments confirm and extend data which demonstrate that hydroxyl radicals are produced by microsomes in the presence of NADPH and oxygen, and in the absence of exogenously added iron.[15] Characteristic DMPO-OH signals were observed when precautions were taken to decrease catalase activity by inhibition with azide (Figure 1), excessive washing (Figure 2), or inactivation by aminotriazole (Figure 2). Formation of azidyl adducts of DMPO when azide was added (Figure 1) is additional evidence for microsomal ·OH formation. The first order rate constant for the reaction of ·OH with azide is about an order of magnitude greater than for its reaction with DMPO,[27] and the azidyl radicals formed are readily trapped by DMPO.[28] The high reactivity of ·OH with azide explains why the DMPO-AZ adducts contribute strongly to EPR spectra, even when azide concentrations are relatively low (e.g. 1 mM, Figure 1). It is also possible that azide could bind iron and facilitate ·OH formation and subsequent azidyl radical generation.

Previous spin trapping experiments designed to test for hydroxyl radical formation in microsomes have been unsuccessful unless a catalytic metal such as

iron,[10,12] or cobalt[13] were also added. It has recently been suggested that DMPO is unsuitable as a spin-trapping agent for detection of ·OH in microsomal systems, because definitive DMPO-OH signals could not be detected.[29] In contrast, DMPO-OH formation has been documented in a highly purified, reconstituted cytochrome P-450 system.[30] The results of the current experiments indicate that addition of SOD and catalase to well-washed microsomes eliminates all EPR signals of ·OH and ·OOH radical adducts of DMPO (Figure 3). Thus, it is likely that contamination of microsomes used in the earlier experiments with catalase, SOD, and possibly other cytosolic components caused the spin trapping experiments to be unsuccessful.

Microsomes generate superoxide during aerobic NADPH oxidation[17] (Table 1), and weak signals from the superoxide radical adduct of DMPO could be detected in well-washed microsomes (Figures 2 and 3). Unfortunately, the superoxide radical reacts with DMPO much more slowly than the hydroxyl radical,[31] and the half-life of the DMPO-OOH adduct is only about one min at physiological pH.[32] For these reasons, spin trapping with DMPO has limited usefulness for detection of microsomal superoxide formation. An alternative method has recently been developed by Rashba-Step *et al.*,[29] in which microsomal superoxide reacts with a hydroxylamine to form a stable nitroxide, which can then be detected by EPR spectroscopy.

Extensive washing increased rates of microsomal superoxide and H_2O_2 formation, NADPH-cytochrome c reductase activity, and the signal intensity of the DMPO-OH adduct (Table 1). However, it was not possible to remove all contaminating catalase or non-heme iron by washing, and the attempt to inactivate residual catalase with AT was also relatively unsuccessful. Sepharose chromatography also failed to remove catalase from microsomes.[15] Because catalase degrades H_2O_2 which may be essential for microsomal ·OH formation, it must be inactivated for optimal spin trapping results. Although azide competes avidly for available ·OH, low concentrations (e.g. 0.1 mM) appear to effectively inhibit catalase activity remaining in well-washed microsomes, and to have little effect on spin trapping experiments. Others have indicated satisfactory results with 0.05 mM azide when α-[4-pyridyl-1-oxide]-N-*tert*-butylnitrone was used as a spin trapping agent.[29]

Iron is most likely the catalytic metal which is involved in microsomal ·OH formation, but its source is still uncertain. The solutions used in these experiments were extensively treated with Chelex resin in order to remove catalytic metals. However, these precautions do not allow exclusion of the possibility that sufficient iron was introduced by buffers or other reagents. On the other hand, the microsomes always contained a small amount of non-heme iron (Table 1), which could not be removed by Sepharose chromatography,[15] and others have shown that this iron pool appears to participate in microsomal lipid peroxidation.[33] Thus, the endoplasmic reticulum itself may contain sufficient amounts of catalytically active iron to stimulate ·OH formation in the presence of H_2O_2.

Finally, the high reactivity of azide with ·OH has not been widely recognized in biological experiments, and could complicate interpretation of results obtained when azide is used as an experimental tool. For example, a number of studies have been conducted to probe the mechanism for microsomal ethanol oxidation.[6,34-36] In such experiments, azide is often added to inhibit peroxidative metabolism of ethanol by catalase. However, because hydroxyl radicals react with azide as well as with ethanol, changes in product formation caused by azide cannot be attributed solely to inhibition of catalase activity.

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