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DIRECT OBSERVATION OF SPIN-TRAPPED CARBON DIOXIDE RADICALS IN HEPATOCYTES EXPOSED TO CARBON TETRACHLORIDE

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Carbon dioxide radical adducts of the spin trapping agent, α-phenyl N-*t*-butyl nitrone (PBN), have been observed to occur in the urine and bile of rats exposed to carbon tetrachloride as well as in perfusates of liver in which the perfusion medium contained carbon tetrachloride (Connor *et al., J. Biol. Chem.*, **261**, 4542, (1986)). The carbon dioxide adduct was proven to be derived from CCl_4 by use of 13-C-labelled compound. These adducts were not observed in the liver itself suggesting that they might be rapidly secreted from the liver. However, using isolated hepatocytes, we have demonstrated that the carbon dioxide radical adduct can be observed directly in the liver cells as it is formed. Since this water-soluble adduct cannot be extracted by non-aqueous solvents such as chloroform or toluene, its formation in liver *in vivo* or in perfused livers was not detected. Lowering the oxygen tension in the system diminished the intensity of production of the carbon dioxide adduct, consistent with the adduct being produced as a result of $\cdot OOCCl_3$ generation. It is not clear the extent to which this adduct is formed as a result of the $\cdot CO_2$ radical or is produced by metabolic oxidation of the trichloromethyl radical adduct of PBN *per se* to the carbon dioxide radical adduct. The intensity of the signal of the carbon dioxide radical adduct suggests that adduct conversion may be the route of formation since it seems unlikely that a sufficient amount of the halocarbon could be metabolized to $\cdot COCl$ or $\cdot CO_2$ radicals to generate a signal of the magnitude involved. The $\cdot CO_2$ adduct is readily observed in intact hepatocytes, but the $\cdot CCl_3$ adduct is not (although we know the $\cdot CCl_3$ adduct has been produced in these cells), indicating that the $\cdot CO_2$ adduct is present in considerable abundance compared to the $\cdot CCl_3$ adduct.

KEY WORDS: Spin trapping, hepatocytes, carbon dioxide radicals, halocarbons, electron spin resonance spectroscopy, free radicals.

INTRODUCTION

Definitive demonstration of free radicals produced during the metabolism of carbon tetrachloride was achieved by the application of the spin trapping technique both *in vitro*[1] and *in vivo*.[2] Metabolism of carbon tetrachloride by rat liver microsomes in the presence of the spin trapping agent, alpha-phenyl N-*t*-butyl nitrone (PBN) and NADPH results in the trapping of the trichloromethyl radical.[3] The resulting PBN spin adduct formed is persistent over a period of hours and can be assayed by electron paramagnetic resonance spectroscopy.[4,5] In addition, administration of PBN either orally or intraperitoneally to rats along with a non-lethal dose of carbon tetrachloride, followed thirty minutes later by extraction of the liver with chloroform-methanol, permits observation of the same adduct in the concentrated extracted material.[6]

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Subsequently, Tomasi and coworkers and also Albano *et al.* demonstrated that spin trapping could be applied to isolated hepatocytes to demonstrate that free radicals were produced during the metabolism of chloroform, bromodichloromethane, bromoform, and iodoform[7] and carbon tetrachloride.[8] All of these procedures involved extraction of the tissues or cells in order to observe the spin adducts.

More recently, Mason and coworkers have shown that when rat livers are perfused with PBN and carbon tetrachloride, carbon dioxide adducts of PBN were observed in the perfusate.[9,10] In addition, urine samples from rats treated with carbon tetrachloride and PBN *in vivo*, also showed the presence of the PBN adduct of CO_2[9,10] as did biliary secretions.[11] The CO_2 adduct was not observed in solvent extracts of the liver of animals treated with PBN and carbon tetrachloride *in vivo*, but if the adduct were produced in the liver, that result would be expected due to the fact that the CO_2 adduct has a negative charge and would not be extracted by a non-polar solvent.[9,10]

In this report we describe the results of experiments with isolated hepatocytes which show: 1) that carbon dioxide radical adducts of PBN can be observed directly in intact, viable hepatocytes which are metabolizing CCl_4 and $CBrCl_3$, but are not observed during the metabolism of $CHCl_3$; 2) that this adduct appears to be situated within the hepatocyte; 3) that the production of the CO_2 radical but not the $\cdot CCl_3$ radical is dependent on the presence of oxygen in the system, and 4) that the $\cdot CCl_3$ radical adduct is not observed in the intact hepatocytes but only in solvent extracts of the cells. To our knowledge, this is the first unequivocal demonstration that spin traps can be used to observe metabolically-generated free radicals directly in living cells.

EXPERIMENTAL PROCEDURES

Freshly isolated hepatocytes were prepared from male Sprague-Dawley rats (150–200 g) as described by Eacho and Weiner.[12] The cells were suspended in Krebs-Hensleit buffer containing 100 mM glucose. Viability of the isolated hepatocytes as determined by Trypan Blue exclusion was greater that 90%, with 80% of the cells still viable at the end of the reactions. The reaction system contained 1×10^8 hepatocytes, 20 mM PBN, 100 μl of the halocarbon to be tested (CCl_4, $CHCl_3$, or $CHBrCl_2$) in a spoon suspended above the incubation medium, and sufficient Krebs-Henseleit buffer containing 15 mM HEPES to make the final volume 10.0 ml. The incubation time was 15 min unless stated otherwise, and the incubation was carried out in a closed, round-botom flask at a temperature of 37°. The halocarbon vaporized from the spoon into the gas phase (either argon, oxygen, or air) during the incubation period, entering the gas phase and then the incubation medium by diffusion. The 100 μl of carbon tetrachloride was sufficient to provide that there would be some of the halocarbon remaining in the spoon at the end of the incubation period.

The $\cdot CO_2$ adduct of PBN was generated chemically using the Fenton reaction and sodium formate. The composition of this system was as follows, 5.0 mM $FeSO_4$, 5.0 mM H_2O_2, and 400 mM sodium formate in 0.15 M potassium phosphate buffer, pH 7.4. The system was incubated for 5 min. The PBN spin adduct of the $\cdot CO_2$ could be detected by direct EPR assay of the reaction system.

Instrument settings were as follows unless indicated otherwise in the data presentations: Receiver Gain: 1.00e + 6; Phase: 0 deg.; Modulation Frequency: 100.00 kHz; Modulation Amplitude: 1.0 G; Conversion Time: 327.69 ms; Time Constant: 327.68;

Sweep Time: 335.544 s; Center Field: 3480.00 G; Sweep Width: 100 G; Frequency: 9.75 GHz; Power: 2.00e + 01 mW.

RESULTS

Hepatocytes were incubated under air in the reaction system containing PBN with CCl_4 vaporizing into the air space as described above. At the end of the incubation period, the cells were sedimented into the sealed capillary end of a Pasteur pipette and placed in the EPR spectrometer so that the cell fraction was in the scanning window. EPR signals derived from these cells consistently displayed the spectrum shown in Figure 1. The coupling constants for this adduct signal are identical to those reported for the $\cdot CO_2$ radical adduct of PBN (a_N = 15.9, a_β^H = 4.6 G).[9] The $\cdot CO_2$ adduct signal was not observed in the incubation medium above the cells. In addition, the $\cdot CO_2$ adduct was not observed in toluene extracts of either the cells or the supernatant medium recovered after the cells were centrifuged into a pellet. On the other hand, even though toluene extracts of the hepatocytes exhibited a reasonably strong signal characteristic of the PBN adduct of $\cdot CCl_3$ (Figure 2), the signal for the trichloromethyl radical adduct of PBN was not observed by direct EPR scanning of these cells.

If ^{13}C-carbon tetrachloride was used in this incubation system, both the $\cdot CO_2$ and $\cdot CCl_3$ radical adducts displayed the twelve-line spectrum that would be expected with the production of $^{13}CO_2$ and $^{13}\cdot CCl_3$ adducts of PBN[10] (data not shown).

If air was purged from systems containing hepatocytes and PBN by flushing with argon for 15 min prior to inserting the CCl_4 container into the gas phase of the reaction vessel, spin trapping studies indicated that very little production of the $\cdot CO_2$ radical occurred (Figure 3B). Allowing oxygen back into the gas phase after 15 min

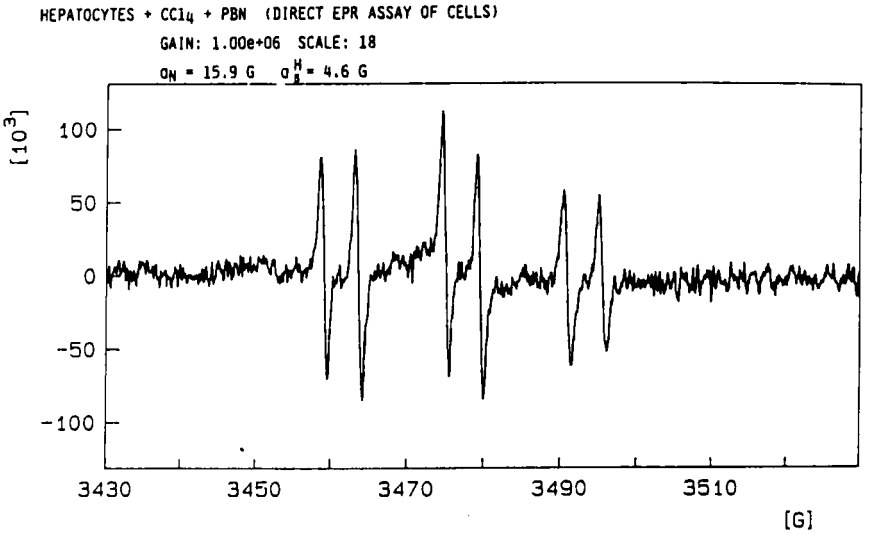

FIGURE 1 EPR signal detected by direct scanning of intact hepatocytes metabolizing carbon tetrachloride under air. The spectrum is assigned to the $\cdot CO_2$ of PBN (a_N = 15.9 G, a_β^H = 4.6 G).

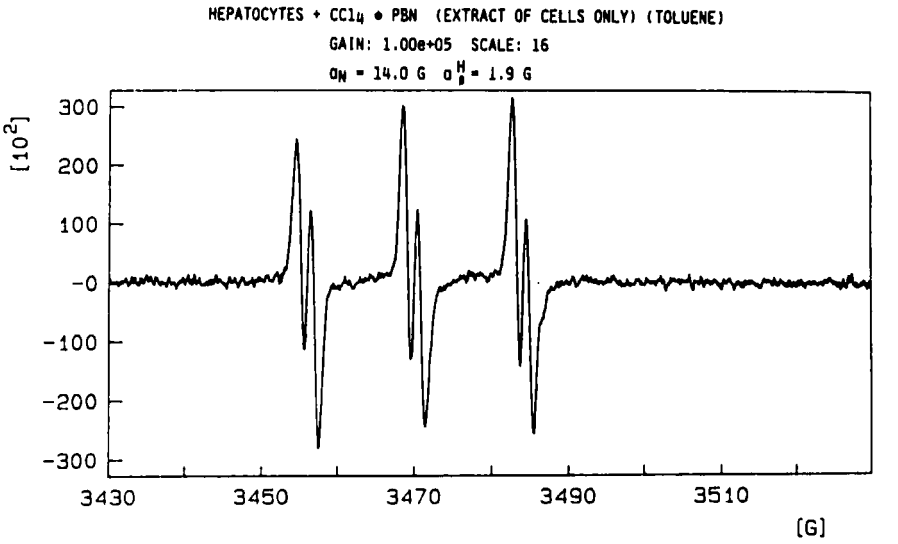

FIGURE 2 EPR signal detected in toluene extracts of hepatocytes incubated for 15 min with carbon tetrachloride. The spectrum is assigned to the $\cdot CCl_3$ adduct of PBN (a_N = 14.0 G, a_β^H = 1.9 G).

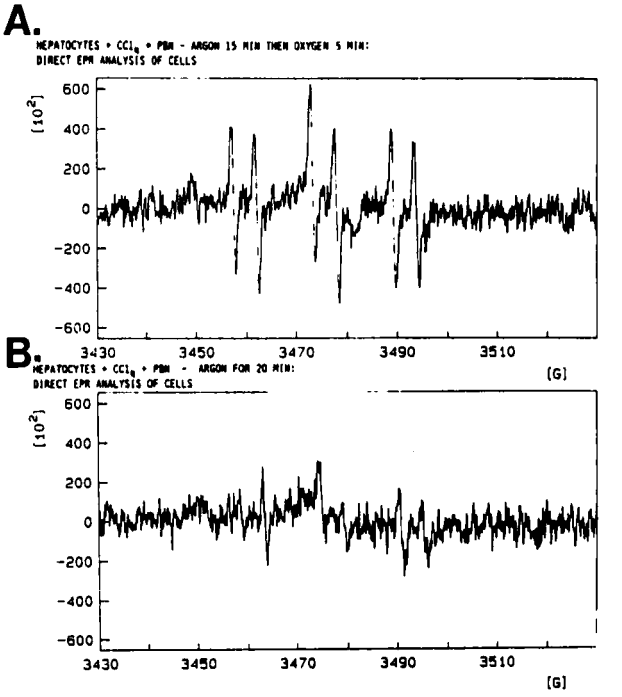

FIGURE 3 A. EPR signal detected by direct scanning of hepatocytes incubated with CCl_4 under an argon atmosphere for 15 min and then under O_2 for 5 min. B. EPR signal detected by direct scanning of hepatocytes incubated with $\cdot CCl_3$ under argon for 20 min.

of incubation under argon followed by an additional 5 min incubation resulted in appearance of the $\cdot CO_2$ radical adduct, although at a weaker intensity than would have been observed if oxygen had been present throughout the incubation period (Figure 3A). These results indicate that incubation under anaerobic conditions did not cause inactivation of the system which produces the $\cdot CO_2$ radical, and that oxygen is apparently required for the generation of this radical. The effect of anaerobic *vs* aerobic conditions on the production of the $\cdot CCl_3$ radical was also significant. The intensity of $\cdot CCl_3$ radical production under argon (Figure 4B) was always greater than production of the radical under oxygen (Figure 4A).

When chloroform was tested in the hepatocyte system, the expected EPR signal for the dichloromethyl radical adduct of PBN was observed ($a_N = 14.4\,G$, $a_\beta^H = 2.2\,G$) in toluene extracts of the cells, but direct observation of the cells for evidence of the $\cdot CO_2$ adduct were consistently negative.

On the other hand, when $CHBrCl_2$ was tested in this cell system, not only was the dichloromethyl adduct observed in toluene extracts of the cells (Figure 5A), but also a weak signal for the $\cdot CO_2$ adduct was detected by direct EPR analysis of the cells (Figure 5B). The toluene extract also showed evidence of the presence of another adduct superimposed on the dichloromethyl adduct signal (Figure 5A).

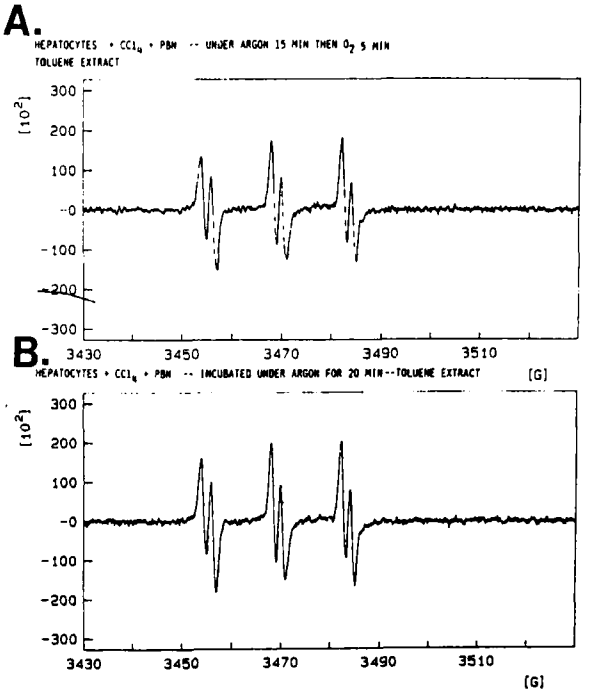

FIGURE 4 A. EPR signal detected in toluene extracts of hepatocytes incubated for 15 min with $\cdot CCl_3$ under an oxygen atmosphere for 5 min ($a_N = 14.4\,G$, $a_\beta^H = 2.2\,G$). B. EPR signal detected in toluene extracts of hepatocytes incubated for 15 min with $\cdot CCl_3$ under an argon atmosphere.

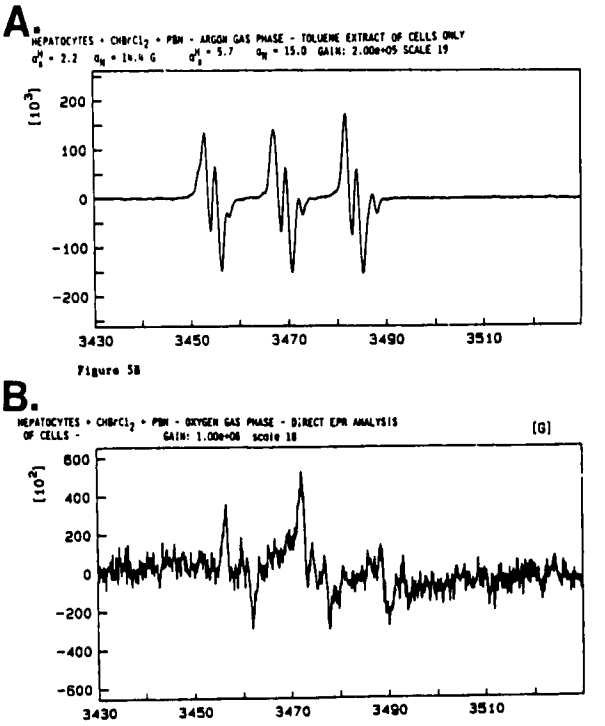

FIGURE 5 A. EPR signal of a toluene extract of hepatocytes incubated with $CHBrCl_2$ under an argon atmosphere for 20 min. B. EPR signal detected by direct scanning of hepatocytes incubated with $CHBrCl_2$ under an oxygen atmosphere for 20 min. The coupling constants approximate those of the $\cdot CO_2$ adduct of PBN ($a_N = 15.9\,G$, $a_\beta^H = 4.6\,G$).

DISCUSSION

The observation of the $\cdot CO_2$ radical in intact hepatocytes before extraction but not in toluene extracts of these cells is consistent with the possibility that this adduct does not partition readily into a non-polar solvent (toluene in this case). On the other hand, the $\cdot CCl_3$ radical adduct was always observed in toluene extracts of the hepatocytes which had been exposed to CCl_4, but not in the direct EPR observation of these hepatocytes. The reason for this seeming paradox may be concerned with the concentration of the adducts present in the hepatocytes at the time of the analyses. A concentration of the $\cdot CO_2$ radical adduct that is considerably higher than that of the $\cdot CCl_3$ adduct might permit detection of the $\cdot CO_2$ by direct EPR observation of the cells without detection of the $\cdot CCl_3$ adduct. In that case, observation of the $\cdot CCl_3$ adducts would not be observed unless the adducts were extracted into a small volume of toluene, thereby increasing their concentration. In view of the fact that only part of the cells in the reaction sytem could be viewed in the cavity (i.e., the cells which were in the capillary section of the pipette that were actually situated within the EPR spectrometer cavity, roughly estimated to be 75% of the cells in the system), it seems clear that considerably more of the $\cdot CO_2$ radical adducts must be produced in the

hepatocytes as compared to $\cdot CCl_3$ radical adducts, at least under aerobic conditions. The $\cdot CO_2$ adduct was not observed in the medium in which the cells were incubated, indicating that most if not all of this adduct is retained in the cells after its formation.

The origin of the $\cdot CO_2$ radical adduct is of considerable interest in that at least two possibilities exist, either of which would have quite different implications for the spin trapping procedure. Under aerobic conditions, a considerable amount of the $\cdot CCl_3$ and $\cdot CHCl_2$ radicals might be expected to react immediately with any oxygen available to form the $CCl_3OO\cdot$ and $CHCl_2OO\cdot$ radicals, respectively. Then, through subsequent conversions of these peroxy radicals by the sequence of reactions (some of which might involve metabolic conversions catalyzed by the cells themselves) may result in production of $\cdot CO^2$ radicals which could then be trapped by PBN. Connor *et al.* have described a possible sequence of reactions for the conversion of the $\cdot CCl_3$ radical to $\cdot CO_2$ via a chlorocarbonyl radical[10]. On the other hand, there is the possibility that the PBN-CCl_3 and PBN-$CHCl_2$ adducts, or the PBN-$OOCCl_3$ and PBN-$OOCHCl_2$ adducts (the latter two being is very unstable),[5] might be metabolized to produce the PBN-CO_2 radical adduct without the $\cdot CO_2$ radical itself ever having been formed. Some of the factors involved in the production of the $\cdot CO_2$ radical adducts must be present in a fraction of the hepatocyte other than the endoplasmic reticulum in view of the fact that in liver microsomal systems metabolizing CCl_4 in the presence of PBN, the $\cdot CO_2$ radical is not observed in direct EPR observations of these systems although strong signals for the $\cdot CCl_3$ radical adduct are seen.

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