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Rat liver microsomal and nuclear activation of methanol to hydroxymethyl free radicals

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Abstract

Recent studies from other laboratories reported that during methanol intoxication lipid peroxidation and protein oxidation in liver occurred. Further, they detected free radicals-PBN adducts in bile and urine of methanol poisoned rats. In this work, we report the presence in liver microsomes and nuclei of NADPH dependent processes of hydroxymethyl (HMet) radical formation. The detection of HMet radicals was performed by GC/MS of the trimethylsilyl derivatives of the PBN (N-*tert*-butyl-a-phenylnitrone)-radical adducts. The formation of HMet radicals was observed only under nitrogen, in these in vitro conditions. Formation of formaldehyde from methanol was observed in aerobic incubation mixtures containing either microsomes or nuclei but also under nitrogen using microsomes. The latter process was not inhibited by diphenyleneiodonium while the anaerobic microsomal one producing HMet was strongly inhibited by it. This shows that they are independent processes. Results suggest that both, liver nuclei and microsomes are able to generate free radicals during NADPH-mediated methanol biotransformation. © 2002 Published by Elsevier Science Ireland Ltd.

Keywords: Methanol; Free radicals; Hydroxymethyl; Microsomal biotransformation; Nuclear biotransformation; Formaldehyde

1. Introduction

Recent studies by Skrzydlewska et al. (2000) reported that during methanol intoxication lipid peroxidation and protein oxidation were observed. The authors postulated that it could be expected that considering the similarity in the chemical structure of methanol and ethanol, free radicals were generated during metabolism (Liesivuori and Savolainen, 1991; Tephly, 1991). The authors provided evidence in their electron spin resonance (ESR) studies of a significant increase in liver extracts from poisoned animals, of a $g = 2.003$ signal attributed by them to free radicals (in general) among others semiquinones (Skrzydlewska et al., 2000). Further, Kadiiska and Mason (2000) detected methanol-derived PBN adducts in bile and urine of PBN-treated methanol

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poisoned rats. Notwithstanding, no report of methanol-derived carbon-centered free radicals formation in liver extracts or subcellular fractions was made in those studies, as it is known to occur in the case of ethanol. During ethanol metabolism in liver microsomes, formation of 1-hydroxyethyl (1HEt) radicals was previously described by others, reviewed by Lieber (1996) and in the highly purified nuclei by our laboratory (Castro et al., 1997, 1998; Díaz Gómez et al., 1999).

In the present studies, we report that during a NADPH-mediated liver microsomal and nuclear metabolism of methanol, hydroxymethyl (HMet) free radicals are formed.

2. Materials and methods

2.1. Chemicals

Methanol (anhydrous) was from Sintorgan, Argentina. Methanol-d_4 was from Merck, Germany. The polyclonal human liver microsomal P450 reductase (P450 Red) (10.0 mg/ml) was from Gentest Corp., USA. Isocitric dehydrogenase (ICDh) from porcine heart, as well as $NADP^+$, NADH, FAD, the spin trap *N-tert*-butyl-α-phenylnitrone (PBN), diphenyleneiodonium chloride (DPI) and dimedone (5,5-dimethyl-1,3-cyclohexanedione) were from Sigma, USA. All the other chemicals used were of the best quality available. Nitrogen (ultra high purity) was from AGA (Argentina) and further deoxygenated by bubbling through a solution containing 0.05% 2-anthraquinone sulfonic acid sodium salt and 0.5% $Na_2S_2O_4$ in 0.1 N NaOH. A standard of the dimethone derivative from formaldehyde was prepared according to Peltonen et al. (1984) and its purity checked by gas chromatography–mass spectrometry (GC–MS).

2.2. Animals

Non-inbred Sprague–Dawley male rats (240–280 g body weight) were used throughout. The animals were starved for 12–14 h before sacrifice. Water was available ad libitum. Animals were killed by decapitation in a Harvard guillotine and their livers were rapidly excised and processed.

2.3. Isolation of microsomes and nuclei

Livers were homogenized in STKM buffer (0.25 M sucrose in 50 mM Tris–HCl/5 mM $MgCl_2$/1 mM KCl, pH 7.5) and highly purified nuclei were obtained as previously described (Castro et al., 1989; Díaz Gómez et al., 2000). The purity of nuclei was assessed on the basis of their lack of activity of marker enzymes for mitochondria (isocitric acid dehydrogenase), for cytosol (lactic dehydrogenase) and by electron microscopy in order to obtain a definitive assessment of the purity of nuclear preparations. These criteria were recently considered as the most suitable to assess that nuclei were free of detectable contamination from other organelles (Rickwood et al., 1997). The hepatic microsomal fractions were isolated by the procedure already reported (Castro et al., 1989, 1990).

2.4. HMet radical generation in chemical model systems

Spin adducts of free radicals arising from methanol were generated in purely chemical reaction systems, in order to obtain mass spectra suitable to select appropriate masses for selected ion monitoring (SIM) detection, necessary in the case of real biological samples. Two model systems were used for this purpose.

2.4.1. Fenton reaction

A 'Fenton system' was prepared essentially as follows (Castro et al., 1997, 1998): 5 mM ferrous sulfate (prepared freshly in N_2-purged water), 5 mM hydrogen peroxide, 0.2 M methanol and 11.2 mM PBN in 50 mM buffer (pH 7.4). Blank samples without any methanol or H_2O_2 were run simultaneously. After addition of ferrous sulfate, the reaction volume was immediately extracted with 500 μl toluene, the organic phase separated and evaporated under N_2 and then silylated with a mixture of bis-(trimethylsilyl)trifluoracetamide (BSTFA):acetonitrile (1:1) and analyzed by GC–MS. Chromatographic conditions were as follows: column, Ultra 1 (crosslinked methyl silicone gum, 25 m × 0.32 mm i.d.), programmed from 100 to 280 °C at a ramp of 10 °C/min. Injection port

was at 250 °C, in the splitless injection mode. Transfer line to MS was at 300 °C. Spectra were taken at 70 eV scanning quadrupole from 50 to 400 amu. Solvent delay was 9.5 min.

2.4.2. FAD–NADPH model system

The ability of the redox system constituted by FAD and NADPH to generate free radicals from methanol was tested (Díaz Gómez et al., 2000). In this case, the incubation mixture contained: 1 mM FAD, 0.2 M methanol and 9.4 mM PBN in 50 mM phosphate buffer, pH 7.4. The incubation vial was purged thoroughly by passing oxygen-free nitrogen previous to the methanol. All reactants were purged with nitrogen and added to the incubation mixture through a septum in order to keep oxygen-free atmospheric conditions. After addition of NADPH generating system (0.45 mM $NADP^+$, 4 mM D,L-isocitric acid trisodium salt and 0.25 units of ICDh), the reaction mixture was incubated 1 h at 37 °C (final volume, 3 ml). Control samples performed in the absence of methanol, or FAD or NADPH generating systems were run simultaneously. The reaction was then extracted with 500 μl toluene, and the organic phase processed as described above. The residue was silylated with BSTFA:acetonitrile (1:1), 60 °C, 15 min and analyzed by GC–MS as above.

Fragmentation pattern in spectra was confirmed by using the deuterated analog of the alcohol (methanol-d_4) and looking for the mass shifts.

2.5. Biotransformation of methanol to HMet radical by human cytochrome P450 Red, liver microsomes or nuclei

The incubation mixture contained human P450 Red (0.1 mg prot./ml), or rat liver microsomes (6.4 ± 0.4 mg prot./ml), or nuclei (4.3 ± 0.3 mg prot./ml), 18.8 mM PBN, 0.2 M methanol and NADPH generating system in a buffered media (50 mM phosphate buffer pH 7.4 in the case of microsomes and P450 Red or STKM for nuclei). Control samples without P450 Red or methanol or NADPH generating system were run simultaneously. When indicated, the incubation vial was thoroughly purged by passing oxygen-free nitrogen previous to methanol. All reactants were purged with nitrogen and added to the incubation mixture through a septum in order to keep oxygen-free atmospheric conditions. When indicated, DPI was present in incubations at 10 μM concentration. After incubating for 1 h at 37 °C under N_2, the reaction volume (3 ml) was extracted with 500 μl toluene, centrifuged and the organic layer evaporated under nitrogen. The residue was silylated with BSTFA and analyzed by GC–MS as above but in the SIM mode, to increase sensitivity. Selected masses were 250 ($M - {}^{\bullet}CH_2OTMS$) and 194 (m/z 250-C_4H_8).

2.6. Biotransformation of methanol to formaldehyde by human cytochrome P450 Red, liver microsomes or nuclei

A 3 ml reaction mixture consisted of: 30 μl human P450 Red (0.1 mg prot./ml) or liver microsomes (4.6 ± 0.3 mg/ml) or liver nuclei (3.4 ± 0.4 mg/ml), NADPH generating system and 0.20 M methanol in a buffered media (50 mM phosphate buffer pH 7.4 in the case of microsomes and P450 Red or STKM for nuclei). When indicated, the incubation vial was thoroughly purged by passing oxygen-free nitrogen previous to methanol. The reaction mixture was incubated for 1 h at 37 °C. Reaction was stopped by placing the flasks on ice and adding 1 ml 20% TCA. Formaldehyde was determined in incubation samples by GC–FID analysis of its dimethone adduct (Peltonen et al., 1984). Briefly, after centrifugation ($1000 \times g$, 5 min, at 4 °C), 1 ml supernatant was added to 0.5 ml 1 N NaOH, 0.5 ml 0.5 N acetic acid and 2 ml 0.4% dimedone. Reaction was allowed to proceed during 30 min at 30 °C, and then samples were extracted with 500 μl toluene. The extract was evaporated under nitrogen and the residue silylated with a mixture of BSTFA:acetonitrile (1:1), 15 min at 60 °C. GC–FID conditions were as follows: column, Ultra 1 (crosslinked methyl silicone gum, 12 m × 0.2 mm i.d.), 230 °C, isothermal. Injection port was at 250 °C, in the split injection mode (1:50). Detector temperature was 280 °C.

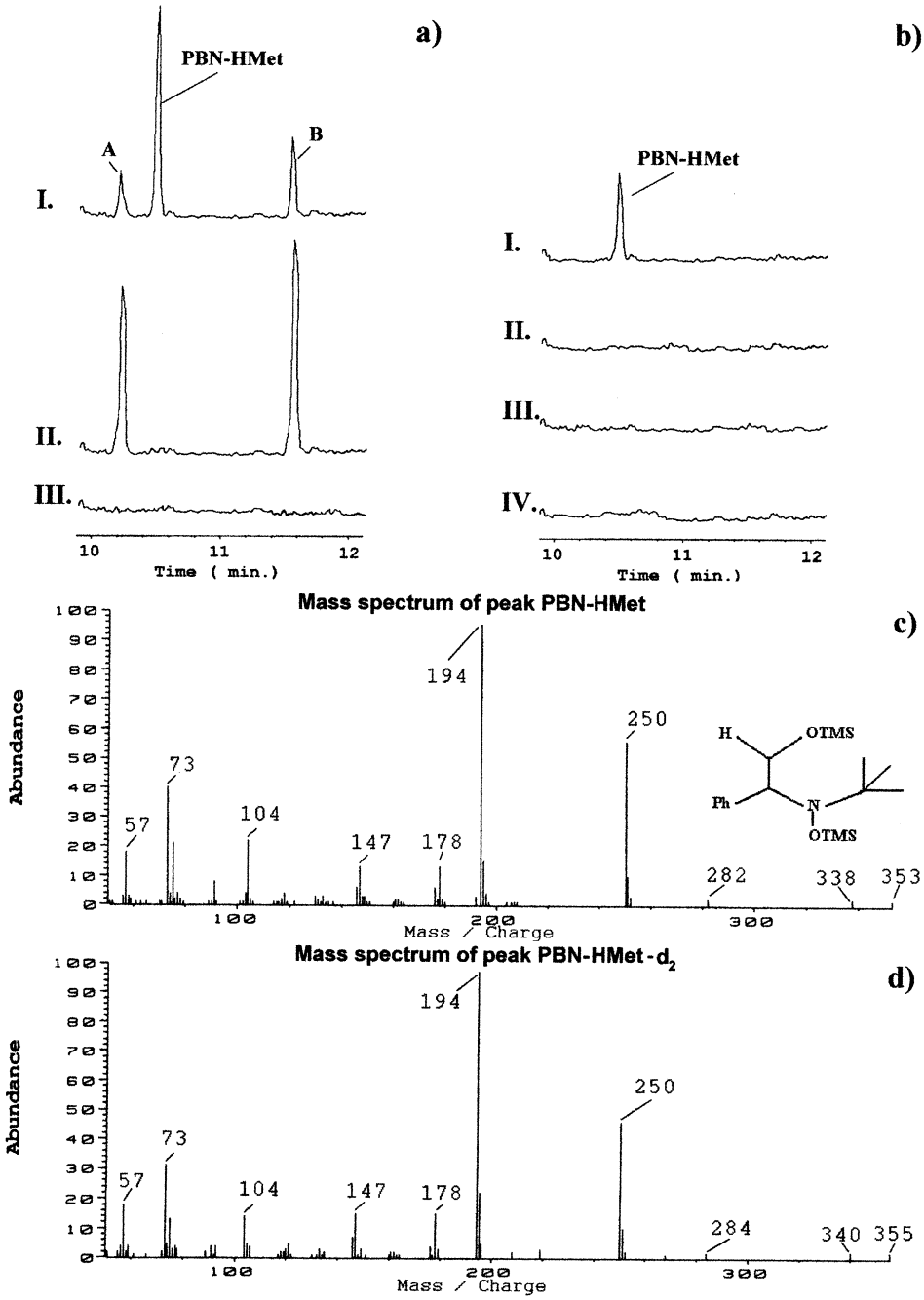

Fig. 1. (a) (I) Gas chromatogram obtained from a sample of the Fenton reaction mixture in the presence of PBN and methanol, after trimethylsilylation. Peaks: A and B, two isomers of hydroxylated PBN; PBN–HMet, HMet–PBN adduct. (II) The same as (I) but in the absence of methanol. (III) Blank without H_2O_2/Fe. (b) (I) Gas chromatogram obtained from a sample of the FAD–NADPH reaction mixture in the presence of PBN and methanol, after trimethylsilylation. (II) The same as in (I) in the absence of methanol. (III) The same as in (I) in the absence of NADPH generating system. (IV) The same as in (I) but in the absence of FAD. (c) Mass spectrum of peak PBN–HMet in Fig. 1a. $M^{+\cdot}$ was observed at m/z 353 and confirmed by fragments m/z 338 (M − 15), m/z 282 (M − 15–56) and confirmed by other ions from a fragmentation pattern, typical of many PBN adducts: m/z 250 (loss of $^{\cdot}CH_2OTMS$ from M), m/z 194 (base peak corresponding to the loss of isobutylene from m/z 250) and m/z 104 (loss of TMSOH from m/z 194). (d) Mass spectrum of peak PBN–HMet-d_2.

3. Results

3.1. HMet radicals generated by model systems

The reaction of methanol with the Fenton reagent or with the reduced FAD system lead to the formation of HMet radicals ($^{\cdot}CH_2OH$) in both chemical model systems tested (Fig. 1a and b). The spectrum of the corresponding PBN adduct (PBN–HMet) is showed in Fig. 1c. Fragmentation pattern was consistent with the presence of a HMet moiety attached to the spin trap structure. Replacement of the alcohol by the deuterated analog methanol-d_4 in the incubation media lead to a spectrum with the expected mass shifts (Fig. 1d). In the case of the Fenton system, other two peaks (A and B in Fig. 1a-I) due to the interaction between hydroxyl radicals and PBN were also observed. No methanol was necessary for them to be formed (Fig. 1a-II). These compounds were previously detected by us in other biological situations and identified as aromatic-hydroxylation derivatives of PBN (Castro et al., 2001; Castro and Castro, 2001).

3.2. HMet radicals generated in different in vitro biological systems

3.2.1. P450 reductase

The pure enzyme was able to generate HMet radicals from methanol under anaerobic atmosphere. The peak detected was the one corresponding to the adduct previously observed to be formed in model systems. Its formation was shown to be strongly dependent on the presence of NADPH (Fig. 2).

3.2.2. Liver microsomes

Results for HMet radical generation in microsomal incubations are depicted in Fig. 3. No PBN–HMet adduct was detected under air, either in the presence or absence of NADPH (Fig. 3a and b). Under nitrogen, the presence of NADPH was necessary for the HMet to be formed (Fig. 3c

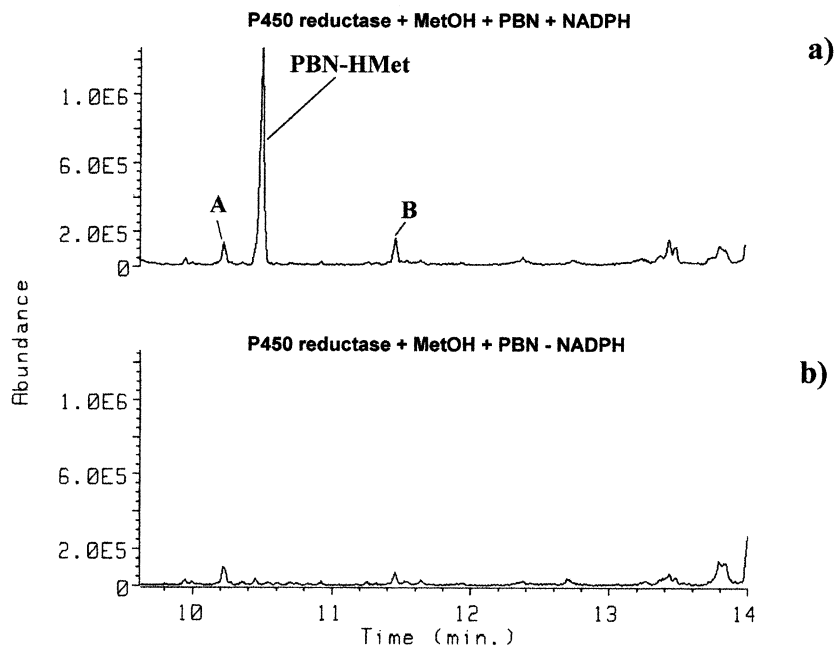

Fig. 2. (a) Gas chromatogram obtained from a sample of incubation containing pure P450 Red, NADPH, methanol and PBN, under nitrogen atmosphere and after trimethylsilylation. Masses selected for SIM were 250 and 194. See Section 2 for details. (b) The same as in (a) but in the absence of NADPH.

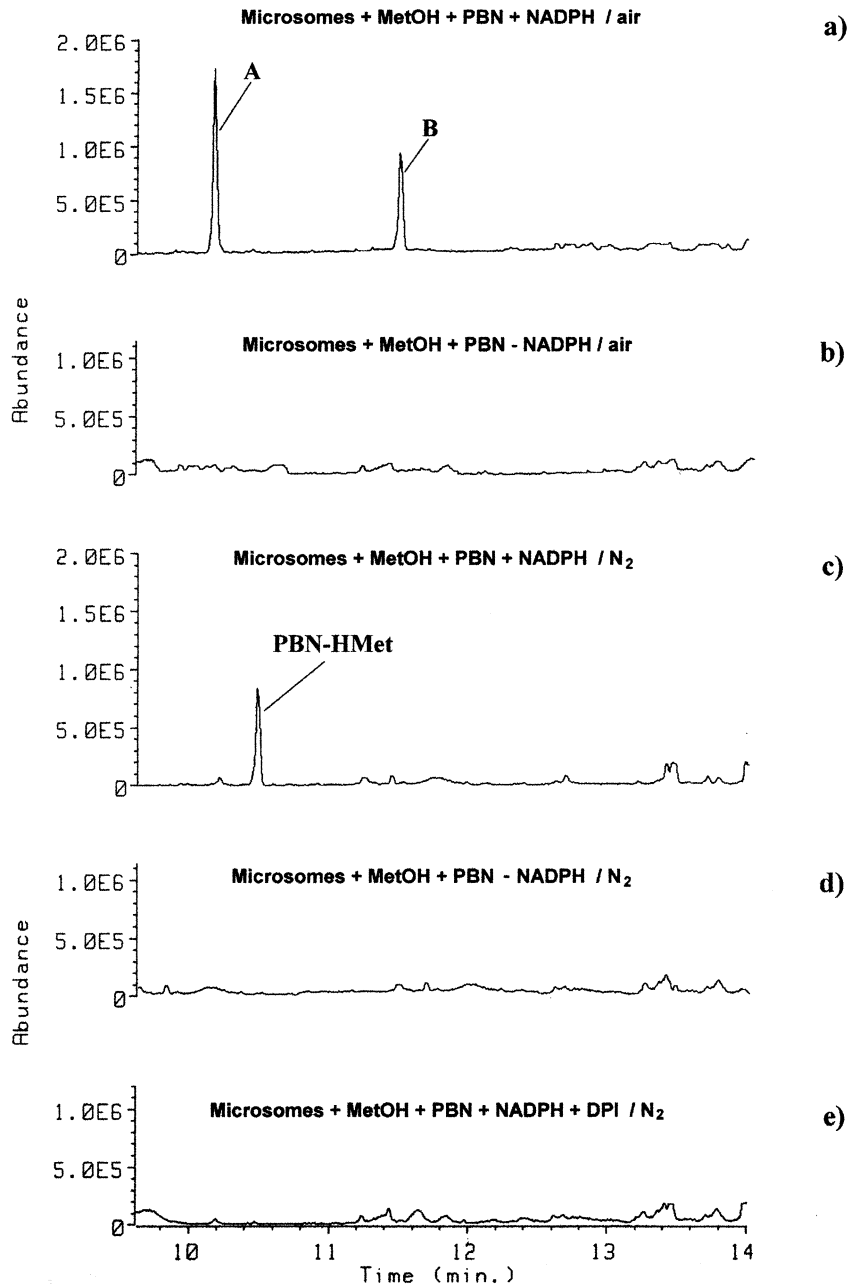

Fig. 3. (a) Gas chromatogram obtained from a sample of incubation containing microsomes, NADPH, methanol and PBN, and after trimethylsilylation. Masses selected for SIM were 250 and 194. See Section 2 for details. (b) The same as in (a) but in the absence of NADPH. (c) The same as in (a) but under nitrogen atmosphere. (d) The same as in (c) but in the absence of NADPH. (e) The same as in (c) but in the presence of 10 μM DPI.

and d) and DPI was able to inhibit all the microsomal biotransformation under those experimental conditions (Fig. 3e).

3.2.3. Liver nuclei

Results for HMet radical generation in incubations containing nuclei are depicted in Fig. 4. No

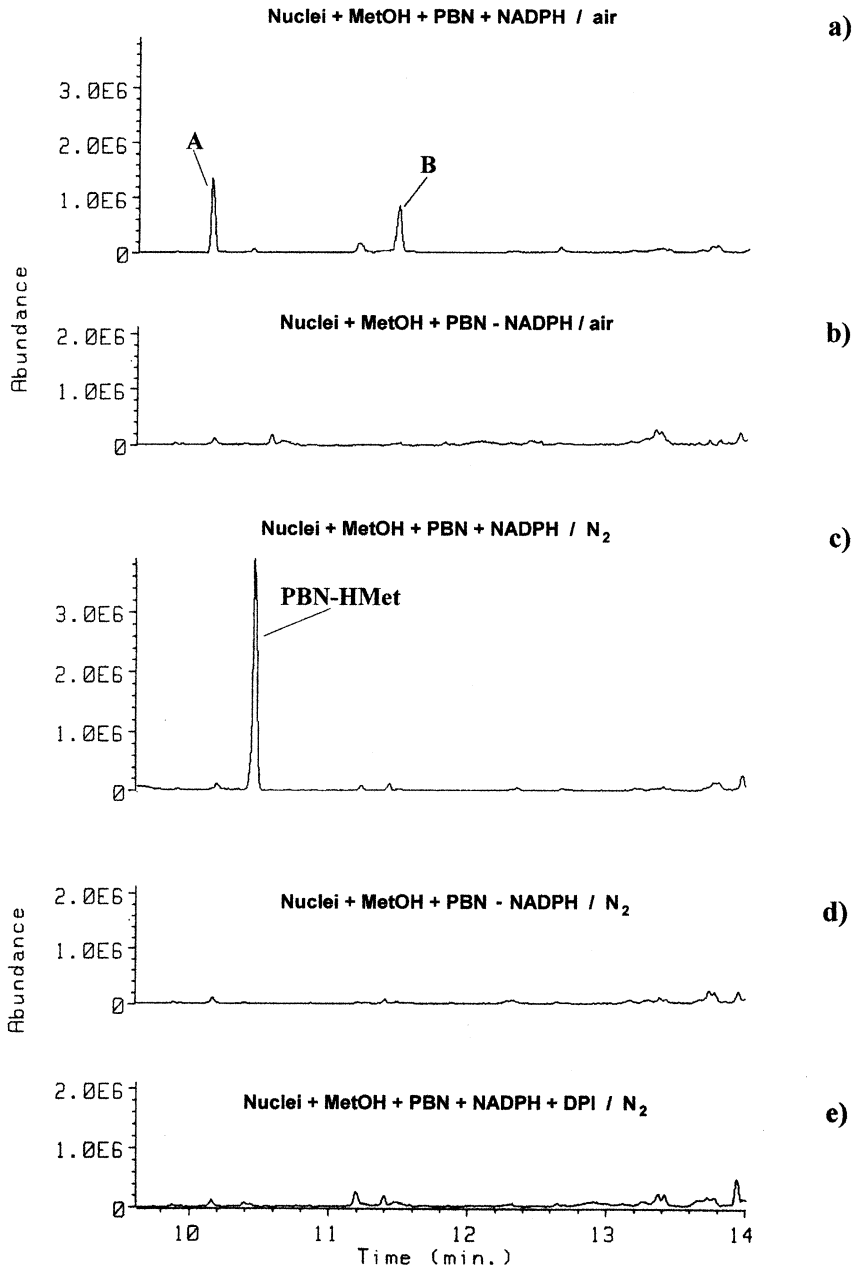

Fig. 4. (a) Gas chromatogram obtained from a sample of incubation containing nuclei, NADPH, methanol and PBN, and after trimethylsilylation. Masses selected for SIM were 250 and 194. See Section 2 for details. (b) The same as in (a) but in the absence of NADPH. (c) The same as in (a) but under nitrogen atmosphere. (d) The same as in (c) but in the absence of NADPH. (e) The same as in (c) but in the presence of 10 μM DPI.

Table 1
Methanol biotransformation to formaldehyde

Experimental[a]	Formaldehyde (nmol)/protein (mg)	
	−NADPH	+NADPH
P450 Red (air)	0	0
P450 Red (N_2)	0	0
Microsomes (air)	0	24.93 ± 1.27
Microsomes (air)+DPI	0	7.54 ± 0.59
Microsomes (N_2)	0	8.60 ± 0.79
Microsomes (N_2)+DPI	0	7.60 ± 0.35[b]
Nuclei (air)	0	7.87 ± 0.63
Nuclei (air)+DPI	0	7.33 ± 0.34
Nuclei (N_2)	0	0

[a] Incubation mixtures containing P450 Red (0.1 mg prot./ml), liver microsomes (4.6 ± 0.3 mg/ml) or liver nuclei (3.4 ± 0.4 mg/ml), in the presence of NADPH generating system and 0.20 M methanol were conducted for 1 h at 37 °C. Formaldehyde was measured as its dimethone adduct, by trimethylsilylation and GC–FID. See Section 2 for details. Each result is the mean of three separate samples.

[b] $P > 0.05$, when compared to microsomes (N_2).

PBN–HMet adduct was detected under air, either in the presence or absence of NADPH (Fig. 4a and b). Under nitrogen, HMet was observed only in the presence of NADPH (Fig. 4c and d) and DPI was able to inhibit all the nuclear biotransformation under those experimental conditions (Fig. 4e).

3.3. Biotransformation of methanol to formaldehyde under different experimental conditions

In order to verify whether P450 Red was able to biotransform methanol to formaldehyde, incubation mixtures were conducted under air and nitrogen atmospheres. Pure P450 Red was not able to biotransform methanol under air or anaerobically, in the presence of the cofactor NADPH (see Table 1).

Liver microsomes, however, metabolized methanol to CH_2O when incubated under air and NADPH. The process was significantly inhibited by DPI. Under a nitrogen atmosphere, the biotransformation to formaldehyde was reduced compared to under air, but still dependent on the presence of NADPH. In this case, DPI was not able to inhibit this metabolism. In the case of nuclei, a small but significant production of CH_2O was detected only in the presence of oxygen and NADPH. No effect for DPI was observed (Table 1).

4. Discussion

In agreement with previous suggestions by Skrzydlewska et al. (2000) and with the results of Kadiiska and Mason (2000) that methanol might be biotransformed to free radicals in liver, now, we describe the presence in both, liver microsomes and highly purified nuclear preparations, of enzymatic processes leading to the formation of HMet radicals. Their formation was specifically and sensitively established by GC–MS of the trimethylsilyl derivative of the PBN–HMet adduct.

The microsomal activation pathway was enzymatic in nature as evident by its inactivation by heating 5 min at 100 °C. The formation of the HMet was only evidenced under anaerobic conditions and it was a NADPH dependent process. Its full inhibition by 10 μM DPI suggest that this anaerobic bioactivation process is mediated by a flavoenzyme, since it is well known that DPI is a specific inhibitor of flavine mediated biotransformations (O'Donnell et al., 1993; McGuire et al., 1998). That possibility is further suggested by our studies where HMet were produced in chemical model systems using reduced FAD, which are useful to gain an insight into the chemical behavior of the flavin moiety. A flavoenzyme potentially being able to perform the observed bioactivation of methanol to HMet might be P450 Red. The likelihood of this working hypothesis rests on the fact that in the present experiments, we found that both reduced FAD and P450 Red are able to generate HMet. Further, in previous studies we reported the ability of NADPH–reduced FAD or the P450 Red to anaerobically bioactivate ethanol to 1HEt radicals (Díaz Gómez et al., 2000). P450 Red enzyme has no known isoforms (Jäning and Pfeil, 1984; Strobel et al., 1995) and it was found to be present not only in the liver endoplasmic

reticulum but also in the nuclear membrane (Kasper, 1971). Accordingly, we also found that highly purified liver nuclear preparations, free of contaminating endoplasmic reticulum (Castro et al., 1998; Díaz Gómez et al., 1999) are also able to biotransform methanol to HMet in a NADPH-dependent anaerobic process that is inhibible by DPI. The formation of highly reactive HMet free radicals near to critical nuclear lipid, protein and nucleic acid components might have additional consequences beyond their quantitative difference with that occurring at more distant cellular locations e.g. the endoplasmic reticulum. We are not in position at present to establish the relevance of that nuclear bioactivation process. We also report in this study pathways of NADPH-dependent biotransformation of methanol to formaldehyde. Formation of formaldehyde from methanol was observed in aerobic incubation mixtures containing either microsomes or nuclei but also under nitrogen using microsomes. The latter process was not inhibited by DPI, while the anaerobic microsomal one producing HMet was strongly inhibited by it. This shows that they are independent processes. The nuclear aerobic formation of formaldehyde was not inhibited by DPI, showing that different enzymes are involved in the nuclear pathway than in the microsomal one. Formaldehyde is also a reactive molecule but far less than HMet and consequently it might have more chances that HMet to travel from production sites at the endoplasmic reticulum to critical target molecules, e.g. in the nuclei. Irrespectively of the detailed knowledge of the enzymatic processes involved, the formation of these two reactive moieties reported here and by others (Skrzydlewska et al., 2000; Kadiiska and Mason, 2000; Lieber, 1996) might help to a better understanding of the liver toxicology occurring during methanol poisoning.

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