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Spin-trapping studies on the effects of vitamin E and glutathione on free radical production induced by 3-methylindole

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3-Methylindole (3MI) is a ruminal fermentation product of tryptophan and causes acute pulmonary edema and emphysema in ruminants when administered orally or intravenously [1]. Substantial amounts of 3MI are also found in the large intestine of man [2] as well as in cigarette smoke as a pyrolysis product of tryptophan in tobacco leaves [3]. 3MI is rapidly metabolised by the microsomal mixed-function oxidase system to produce a nitrogen-centered 3MI free radical which initiates lipid peroxidation causing the formation of carbon-centered radicals from microsomal membranes in the lung [4]. Although the exact mechanism of 3MI toxicity is not known, a free radical produced from 3MI may be of significance. Vitamin E and glutathione (GSH) have been implicated as an important defense against free radical injury by sequestering free radicals. In our previous work [4], we have shown that vitamin E and glutathione (GSH) inhibit formation of the 3MI nitrogen-centred free radical and of the carbon-centered lipid radicals in *in vitro* lung microsomal preparations. The objective of this study is to investigate the effects of vitamin E and administration of cysteine (CYS) and diethylmaleate (DEM) on the levels of free radicals generated *in vivo* by 3MI metabolism in the intact goat using spin-trapping techniques.

Materials and methods

Chemicals. 3MI, DEM and GSH (reduced form) were obtained from the Sigma Chemical Co. 4-Oxo-2,2,6,6-tetramethylpiperidinooxyl was purchased from the Aldrich Chemical Co. DL-α-Tocopheryl acetate was obtained from the Grand Island Biological Co. The spin trap, PBN (α-phenyl-*tert*-butyl nitrone), was available in the laboratories of the Department of Chemistry and Biochemistry, University of Guelph, but can be purchased from the Sigma Chemical Co. CYS hydrochloride was obtained from the Fisher Scientific Co.

In vivo *study*. The *in vivo* spin-trapping experiments used sixteen male, weanling goats which were fed a 12% protein diet [5] *ad lib.* and had free access to water for 2 weeks. Animals were housed in pairs in galvanized steel pens with perforated floors in a room with a 12-hr photoperiod and maintained at 23°. The goats were divided into four groups: (1) vitamin E + CYS, (2) vitamin E + DEM, (3) CYS, and (4) DEM. CYS, the precursor of tissue GSH, was administered intraperitoneally as L-cysteine hydrochloride (0.40 g/$W^{0.75}_{kg}$); body wt to 0.75 power = metabolic body wt) and vitamin E was given in corn oil intramuscularly (130 I.U./$W^{0.75}_{kg}$) once every 24 hr for 3 consecutive days. On day 4, the animals were challenged with 3MI in propylene glycol at a dose of 0.02 g 3MI/kg and 0.10 g PBN/$W^{0.75}_{kg}$ by intrajugular infusion at a rate of 0.05 to 0.06 ml/min for 1.0 hr using a peristaltic pump (Brinkmann Instruments). DEM (0.2 ml/$W^{0.75}_{kg}$) was given by a single intraperitoneal injection 1 hr before the infusion began to deplete tissue GSH levels [5]. Control animals received the PBN-propylene glycol emulsion but were not exposed to 3MI. All goats were killed immediately after the 1 hr infusion by captive bolt gun, and the lungs and liver were immediately removed, weighed and frozen in liquid N_2. The tissues were homogenized in distilled water (1:1), and the spin adducts were extracted two times with an equal volume of hexane. The extract was concentrated by rotor evaporization and dried with anhydrous sodium sulfate. The samples were gassed with N_2 for 15 min before ESR analysis to eliminate O_2 from the samples. The ESR spectrum of the extracted, spin-trapped radicals was recorded at room temperature with a Varian E-104 spectrometer. The instrument settings were: microwave power, 20 mW; modulation amplitude, 1G; time constant, 1S; scan range, 100G; and scan time, 8 min. A quantitative estimation of the relative amounts of PBN-trapped free radicals in the lung of intact goat after a 1-hr exposure to 3MI was made by the method of Ayscough [6], employing the nitroxide free radical standard, 4-oxo-2,2,6,6-tetramethylpiperidinooxyl.

In vitro *study*. Microsomes were prepared from livers of mature male goats (without pretreatment) according to methods described earlier [4]. The microsomes were resuspended in the phosphate buffer (0.01 M, pH 7.4) used throughout the experiment described herein. Protein concentrations were determined according to the method of Lowry *et al.* [7]. The reaction mixtures contained 48 mg of microsomal protein, 0.1 M PBN, 0.1 mM EDTA, 0.2 μM NADPH, 0.063 M 3MI in a total volume of 6 ml of 0.01 M phosphate buffer (pH 7.4). The 3MI was first dissolved in ethanol (2% of the final volume). The reaction mixtures were preincubated at 37° for 5 min before the addition of 3MI. In addition, 0.5 mM DL-α-tocopherol or 0.05 M GSH was added in the incubation system. The incubation mixture was stopped at 5 and 60 min after the addition of 3MI by extraction with hexane. Spin adducts formed in the incubation mixtures were extracted twice with 6 ml of hexane, then dried with anhydrous sodium sulfate, and concentrated under a stream of N_2 to 450 μl. The ESR spectra were recorded under the same setting as in the *in vivo* study.

Results

In the present study, four goats in each group were given vitamin E + CYS, vitamin E + DEM, CYS and DEM treatments prior to the intrajugular infusion of 3MI. The ESR spectra obtained from the hexane extract of the lungs after the various treatments are shown in Fig. 1. Only one of the four goats of the vitamin E + CYS treatment group showed the ESR signal seen in Fig. 1A; no ESR signal was detected in the other three goats. In the vitamin E + DEM group, three out of four goats had the ESR signal shown in Fig. 1B; no ESR signal was detected in the lung of the fourth goat. Figure 1C shows the typical ESR signal obtained from all four goats in the CYS group. Figure 1D shows the typical ESR signal seen from all four goats receiving DEM treatment. The ESR spectrum of the PBN spin adduct obtained from the above treatments is due to a carbon-centered radical adduct with hyperfine splitting constants of a_N = 14.4 G and a_β^H = 3.2 G. The ESR signal was not observed in the hexane extract of liver tissue of goats following administration of 3MI regardless of pretreatments (Fig. 1E). In addition, administration to goats of the spin-trapping agent in propylene glycol without 3MI, followed by hexane extraction, yielded an extract that produced no measurable ESR signal in either the lungs or liver. Hence, the spin-trapped radical was not formed in the goat lung *in vivo* in the absence of 3MI administration.

The calculated amounts of free radicals in the extracted lung, expressed as picomoles of PBN-trapped radicals per lung, are shown for the various pretreatments in Fig. 2. The vitamin E + CYS group and the CYS group had the lowest concentrations of the trapped radical. However,

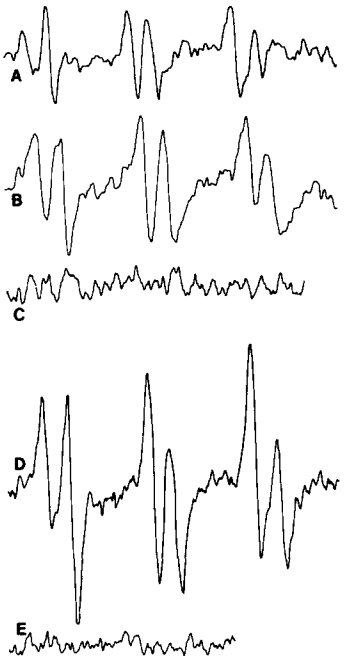

Fig. 1. (A) ESR signal of a hexane extract from lung of a goat given vitamin E + CYS treatment prior to infusion with 3MI and PBN. (B) ESR signal of a hexane extract from lung of a goat given vitamin E + DEM treatment prior to infusion with 3MI and PBN. (C) ESR signal of a hexane extract from lung of a goat given CYS treatment prior to infusion with 3MI and PBN. (D) ESR signal of a hexane extract from lung of a goat given DEM treatment prior to infusion with 3MI and PBN. (E) ESR spectrum showing the absence of an ESR signal in liver hexane extract of goats infused with 3MI and PBN.

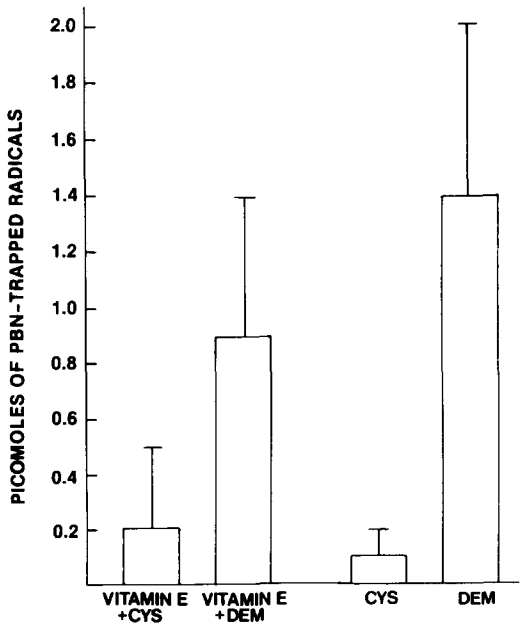

Fig. 2. Calculated amount of free radicals from ESR spectra of extracted lung expressed as picomoles of PBN-trapped radicals per lung (mean ± S.E.).

when tissue GSH levels were depleted with DEM pretreatments, higher concentrations of the PBN-trapped radical were detected regardless of vitamin E pretreatment.

Figure 3 shows the ESR spectra obtained from liver microsomes incubated with 3MI, NADPH, and PBN for 5 and 60 min. After a 5-min incubation, a nitrogen-centred free radical with hyperfine splitting constants of a_N = 13.9 G, a_β^H = 3.6 G and a_β^N = 2.3 G was obtained (Fig. 3A). After a 60-min incubation, a free radical with hyperfine splitting constants of a_N = 14.4 G and a_β^H = 3.2 G characteristic of a carbon-centered free radical [8, 9] was obtained (Fig. 3B). Although identification of the carbon-centered radical adduct has not been made, the magnitude of the a_β^H splitting constant suggests that it is probably a small alkyl group [8, 9]. These signals are identical to those that were obtained when 3MI was incubated with lung microsomes [4]. Figure 3C and 3D show the inhibition of the carbon-centred free radical adduct by the addition of 0.5 mM vitamin E or 0.05 M GSH in the same incubation system as in Fig. 3B.

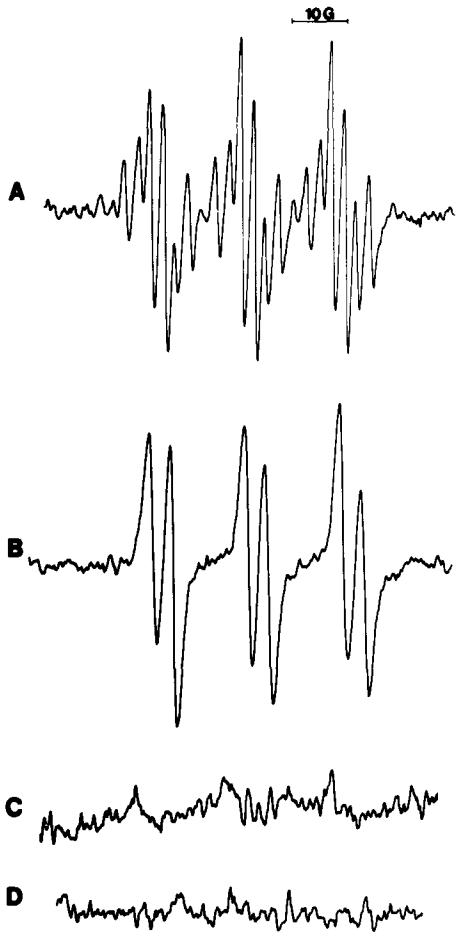

Fig. 3. (A) ESR spectrum of a liver microsomal incubation system containing approximately 16 mg protein/ml, 0.1 M PBN in 0.05 M phosphate buffer (pH 7.4), 0.063 M 3MI, and 0.2 μM NADPH. Incubation was stopped after a 5-min incubation period at 37°. (B) Scan of A after a 60-min incubation period. (C) ESR spectrum obtained upon the addition of 0.5 mM vitamin E in the same incubation described in B. (D) ESR spectrum obtained upon the addition of 0.05 M GSH in the same incubation described in B.

Discussion

In the present study and in our previous work [4], evidence has been shown that lipid radicals are generated in the metabolism of 3MI in the goat lung *in situ* (Fig. 1). The radical that was formed *in vivo* has the same splitting constants as the carbon-centered radical formed by the metabolism of 3MI of lung *in vitro* which was demonstrated to be a lipid peroxidation product of microsomal membrane [4]. These results thus indicate that 3MI causes lipid peroxidation in goat lung *in vivo*. Lipid peroxidation has been strongly implicated in pulmonary injury resulting from exposure to a number of pulmonary toxicants [10, 11].

Liver microsomes were shown to be capable of metabolizing 3MI to the nitrogen-centered 3MI free radical (Fig. 3A) and subsequently causing the formation of carbon-centered lipid radicals (Fig. 3B) as observed previously with lung microsomes [4]. Furthermore, the formation of the carbon-centered radical adduct was found to be inhibited by the addition of GSH and vitamin E to the liver microsomal incubation system, as has been earlier shown with lung microsomes [4]. However, unlike the lung, the liver showed no detectable ESR signal upon infusion of 3MI and PBN in the *in vivo* study. PBN has previously been used *in vivo* to trap free radicals generated from liver metabolism of halothane [12] and CCl_4 [13]. This lack of an ESR signal in the liver is not due to an unequal distribution of 3MI between lung and liver as Bradley and Carlson [14], showed that the amounts of 3MI that reach the lung and liver after infusion are approximately the same. The absence of an ESR signal in the liver may, therefore, be due to higher concentrations of GSH and other antioxidant defense systems in the liver preventing the formation of secondary carbon-centered radicals from 3MI metabolism. The inhibitory effect of GSH and vitamin E on carbon-centered radical formation in *in vitro* liver microsomal incubations of 3MI suggests this possibility.

Vitamin E and GSH have been implicated in the protection of membrane lipids from peroxidative damage initiated by free radical processes [15, 16]. The very high dose of 130 I.U. DL-α-tocopherol/kg of metabolic body weight was used in the *in vivo* study to see whether further protection would be provided to goats by more vitamin E than is normally acknowledged to be adequate for non-toxic stresses. In relation to lipid radical production, any extra protection provided by the large dose of vitamin E was marginal. The lack of protection by the vitamin E pretreatment may have been due to the vitamin E dose not reaching the lung tissue to significantly elevate the vitamin E content. This possibility requires further study. In addition, vitamin E has been shown to react poorly with carbon-centered lipid radicals but react rapidly with oxygen-centered peroxy radicals [17]. Thus, the effect of vitamin E on free radical concentrations may have been missed as its main action as a free radical scavenger of oxygen-centered free radicals was not measured in this study.

Our results show that, in the *in vivo* system, the susceptibility to peroxidation in lung tissue correlated with treatments which increased or decreased lung tissue GSH content. It is known that CYS is the precursor of GSH synthesis [18] and that DEM depletes GSH in many organs including the lung [19]. The dose of DEM was equal to that used previously in goats to deplete lung GSH and increase the severity of pneumotoxic effects of 3MI [5]. Conversely, the dose of CYS used in this study has been shown to significantly elevate GSH in the liver and lungs of goats was well as to protect goats against 3MI-induced pulmonary disease [5]. GSH may exert its protective role by reacting directly with the nitrogen-centered free radical of 3MI, thus preventing its covalent binding to cell macromolecules [14] and/or the initiation of lipid peroxidation. The results of this study support the view that lipid peroxidation is involved in 3MI-induced pulmonary toxicosis and that tissue GSH plays an important role in the lung defenses against 3MI-mediated lipid peroxidation.

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