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WHOLE MOUSE NITROXIDE FREE RADICAL PHARMACOKINETICS BY LOW FREQUENCY ELECTRON PARAMAGNETIC RESONANCE

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The *in vivo* uptake distribution and reduction of the oxygen-sensitive nitroxide spin label PCA in the mouse monitored by low frequency electron paramagnetic resonance (EPR) spectroscopy are reported. Spectra were obtained from the head and liver regions of pentobarbital anesthetized mice during different circulatory and ventilatory conditions. Identical clearances were found in these regions during normoxia. Moderate hypoxia (10% O_2-90% N_2) did not significantly affect the spin label reduction rate.

Oxygen-centered free radicals have been implicated in a variety of cellular events including reperfusion injury, cancer and aging (1). Until recently, the only conventional electron paramagnetic resonance (EPR) spectroscopic technique available to measure free radicals has been spin trapping (2). However, more recently low frequency EPR has been applied *in situ* to measure nitroxide free radical pharmacokinetics in intact mice (3) and isolated rat hearts (4,5) as well as to visualize the rat tail using a nitroxide solution as EPR contrast agent (6). Currently, nitroxides are under active investigation both as imaging agents for *in vivo* EPR techniques and as contrast agents for nuclear magnetic resonance (NMR) imaging. In this last application, nitroxides have a number of attractive properties including chemical versatility and low toxicity (7-9). Another important feature of nitroxides is their spectral sensitivity to the oxygen concentration in biological tissues. The oxygen/nitroxide interaction shortens the relaxation times, inducing line broadening (10). In tissue and cell suspensions, nitroxides are reduced to their corresponding hydroxilamines which are then reoxidized to nitroxides at a rate depending on the oxygen tension (7). Oxygen affects the metabolism of these nitroxides primarly by interacting with cytochrome c oxidase and changing the redox state of the enzymes in the respiratory chain (11). The dependance of nitroxide metabolism on oxygen concentration

ABBREVIATIONS

EPR: electron paramagnetic resonance; NMR: nuclear magnetic resonance; PCA, 2,2,5,5 tetramethylpyrrolidine-1-oxyl-3-carboxylic acid.

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could be a valuable tool in measuring the actual oxygen concentration during *in vivo* EPR and NMR experiments.

The purpose of this study was to obtain EPR spectra from selected regions of anesthetized mice and to compare the pharmacokinetics of an oxygen sensitive pyrrolidine nitroxide (2,2,5,5- tetramethylpyrrolidine-1-oxyl-3-carboxylic acid: PCA) in different organs during normoxia and moderate hypoxic hypoxia of these animals. The PCA spin label was chosen for its low toxicity, high *in vitro* reduction rate in anoxia (7) and low enzyme dependent oxidation from the correspondent hydroxilamine (9).

MATERIALS AND METHODS

Low frequency EPR Spectrometer. The low frequency EPR spectrometer was composed of a conventional design reflex bridge utilizing a Hewlett Packard (Palo Alto, CA) model 8640B sweep oscillator as microwave source and a three-arm re-entrant resonator as sample cavitiy which was recently designed (12). The power level was 100 mW. Experiments on mice tails were performed at a microwave frequency of 1.2 GHz utilizing a resonant cavity with a sample insertion diameter of 12 mm. Experiments on whole mice were performed at 680 MHz utilizing a cavity with an access diameter of 24 mm. The sensitivity of the spectrometer at both frequencies was determined using sample tubes with different PCA concentrations. The minimum detectable concentration in physiologic solution was $5x10^{-6}$ M. A five fold sensitivity has recently been reported for aqueous solutions (4). The sensitivity is proportional to the value of the cavity quality factor Q, i.e., 2π times the radiofrequency energy stored in the resonator cavity / the energy dissipated for each radiofrequency cycle. We obtained a lower sensitivity because we employed spin label diluted in saline solution instead of aqueous solution. The presence of ions, in fact, produced magnetic field losses. During *in vivo* experiments animal movements caused a spectral noise coherent with respiration. However, the frequency of this noise was well defined and could be filtered by using discrete Fourier transformations and cutting off the noisy frequencies.

In vivo studies. Mice weighting 18-22 g were anesthetized by pentobarbital (40 mg/kg, intraperitoneally). Pyrrolidine PCA (Sigma Chemical Co, St. Louis MO) with a partial negative charge was employed in these studies. The pharmacokinetics, metabolic behavior and oxygen-dependent reduction rate of this nitroxide by mammalian cells has been already reported (7-9,13).

The spin label-buffer solution was injected into the tail vein or, in some experiments, intraperitoneally at a dose of 0.33 mM/kg. The animal was restrained by a soft polyethylene tube which was quickly inserted into the EPR cavity. Organs under examination were positioned at the center of the active region of the cavity (35 mm in length). For instance, in order to obtain measurements from the liver, the liver region was placed in the middle of the active region of the cavity. Head and liver pharmacokinetics from the same animal could also be observed by moving the sample holder inside the cavity to fixed positions.

The pharmacokinetics of the PCA nitroxide were investigated during different experimental conditions. Experiments were conducted by using one of the following four protocols: (a) PCA clearance from single regions (head and liver) during air ventilation eventually followed by hypoxic hypoxia (10% O_2-90% N_2), (b) comparison of PCA clearance measured from head and liver of the same animal during air ventilation, (c) PCA clearance from the head during air ventilation followed by hemorrhage-induced hypothension produced by bleeding the animal from the tail artery, and (d) PCA clearance in the head and tail during air ventilation followed by animal sacrifice (decapitation and drainage of the residual blood from these organs by gravity). Repetitive EPR acquisitions were conducted for 50-70 min in all the above protocols after administration of nitroxide. The peak to peak amplitude from the triplet central line of a filtered spectrum was taken as a measure of signal intensity. Clearance curves could be fitted by a monoexponential function. In order to quantitate the rate of nitroxide clearance, data were plotted in semilog scale where a straight line was obtained. From its slope the PCA half life was calculated.

RESULTS

Uptake and clearance of PCA in different regions of the mouse after bolus injection were studied. Prior to PCA injections, no EPR signal was observed. After intravenous injection, the PCA triplet signal appeared immediately. The spectra obtained from the PCA present in a phantom and in a mouse head are shown in Fig. 1. The mouse movements caused a deterioration of the signal to noise ratio more evident in the head then in other regions. This was caused by the larger mobility of the head in the sample holder. By proper filtering of the experimental data, described in methods, this effect could be largely removed. The resulting spectrum, superimposed on the untreated one, is shown in Fig. 1 B. After the intravenous injection, the triplet amplitude quickly decreased in the head as well as in the other monitored regions. Half life in the head was 13.5 ± 3 min (mean $\pm$ standard deviation) for four animals.

The resulting spectra are a composite of local spectra from different regions of the observed volume and are therefore strongly dependent on the concentration profiles of the nitroxides in the parenchyma and in the body fluids. In order to verify the contribution of blood to the tissue EPR signal, two animals were subjected to hemorrhagic hypotension 20 min after spin label injection in order to drastically decrease volemia. Although the animals lost about 60 % of the blood estimated to be present, the PCA triplet amplitude measured on the head before and after hemorrhage was unchanged. Spectra were also obtained from the head and tail after the

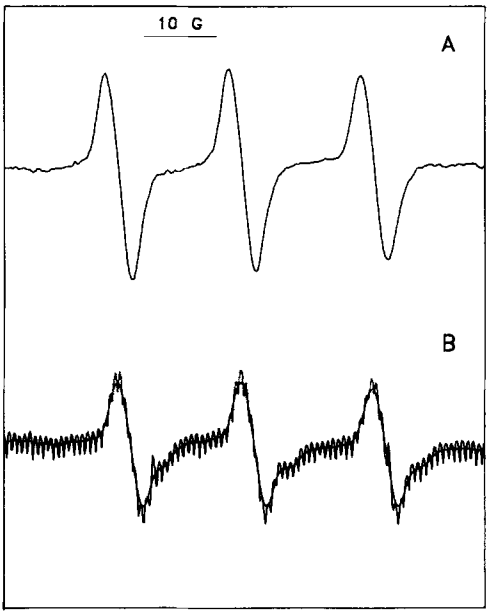

Figure 1.
Spectra obtained from 5×10^{-6} M PCA present in a phantom (A) and in a mouse head 2 min after a 0.33 m M/kg spin label intraperitoneal injection (B) (100 sec scanning time, 400 msec time constant, and 100 mW radiofrequency power level). The 20 mm phantom tube was filled with the typical mouse spin label dose diluted in physiologic solution. Spectrum B shows regular oscillations due to the mouse rhytmic respiratory motions. This noise was eliminated by the use of digital filtering and the resulting spectrum is superimposed.

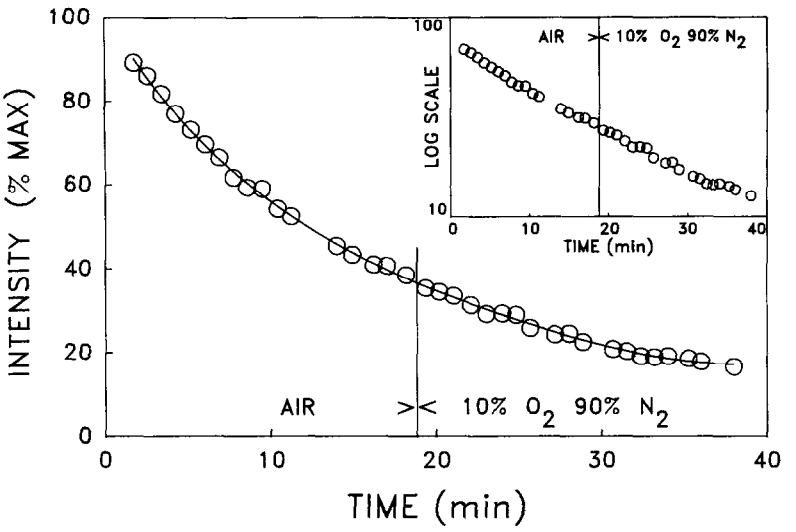

Figure 2.
Typical clearance curve of PCA nitroxide from the head of a mouse after a 0.33 mM/kg spin label intraperitoneal injection during 20 min air ventilation followed by a 10% O_2 -90% N_2 gas mixture. Signal intensity was measured from filtered spectra from the peak to peak amplitude of the triplet central line. Spectra were collected sequentially with a field window which included only the triplet central line. Scanning time was 40 sec, time constant 400 msec and power level 100 mW. The inset shows data plotted in semilog scale. PCA half lives, calculated from the slope of the straight line, were 13.2 and 14.6 sec in normoxia and hypoxia, respectively.

animals were sacrificed by decapitation 20 min after PCA injection and the residual blood removed from these organs by gravity. In this experimental condition, the spin label was unchanged in the blood-free head and tail . These data suggest that 20 minutes after nitroxide injection most of the PCA EPR signal originated from the parenchyma.

In order to understand the PCA uptake and clearance mechanism, liver and head were compared after PCA injection in the perivascular space of the caudal vein.. The triplet was detected in both organs after 4-6 min and the triplet amplitude increased in the same manner reaching the maximum value after 13-17 min in both regions. This suggested that no differential uptake mechanism was present in these organs. In the following minutes, triplet amplitude decreased in both organs and the clearances were not significantly different. A similar uptake and clearance were found in separate experiments in which the lower abdomen was monitored. PCA, in fact, is excreted by the kidneys at the rate of the glomerular filtration (14) and is concentrated in the bladder. Therefore, at least in this experimental animal model, kidney PCA clearance was a relevant factor to be considered together with tissue metabolic factors in the analysis of regional clearances. Similar uptake and clearance kinetics were observed in the same body regions when the nitroxide bolus was injected intraperitoneally.

In order to evaluate the head reduction rate during moderate hypoxia, two animals were given a 10% O_2 -90% N_2 gas mixture to breathe 20 min after room air ventilation (Figure 2). A moderate increase (11 and 17 %) could be detected when the rate of nitroxide clearance during normoxia and subsequent hypoxia were compared.

DISCUSSION

The results of the present study show that low frequency EPR spectroscopy can be used to examine the pharmacokinetics of PCA nitroxide radical metabolism in different regions of the mouse. Several groups are developing instruments for this purpose (3,15). To our knowledge, this is the first paper reporting spin label EPR spectra of the whole head of the intact mouse.

The rate of reduction of PCA *in vitro* has been reported to be a function of oxygen concentration. The possibility of detecting this spin label probe in intact tissues by EPR spectroscopy and/or imaging might provide a powerful tool to discriminate among normoxic, hypoxic and anoxic tissue conditions in different experimental animal models. For these reasons, the reduction of nitroxides *in vivo* and *in vitro* is the subject of active investigation. Nitroxides are reduced to their corresponding hydroxilamines when administered intravenously. This one-electron reduction can be mediated by a number of different cellular reductases including ascorbic acid. Recent data, however, suggest that ascorbic acid can account for only 5% of the reduction of PCA in rat liver homogenates (16). The major variables that affect the rate of reduction may include the type of tissue or cell, the lipid solubility of the nitroxide, the ring structure of the nitroxide, temperature, oxygen concentration and pH (17). PCA reversibly ionizes at pH 7 and is expected to be present mainly in acqueous enviroments. During PCA transport to tissues nitroxide can be reduced by the blood reducing agents. The rate of penetration of nitroxide into cells is a second factor of potential importance in understanding the reduction rates of PCA. *In vitro* experiments have shown that intracellular concentration of PCA in bone marrow cells rises for about 10 min and then begins to decrease (8). This suggests that the *in vivo* kinetics of spin label reduction are highly complex, dependent upon the blood reducing capacity, cellular uptake and cellular reducing capacity and above all renal clearance. Kidneys cleared PCA from the circulation at the glomerular filtration rate (14) and the spin label was reduced by urine enzymes (3).

Decreasing volemia by hemorragic hypotension and quick blood removal after animal sacrifice 20 min after the PCA injection demonstrated that the EPR signal originated mostly from the parenchyma. Consequently, at this time, the reduction rate was probably due to cellular factors. In addition, we found the same PCA clearance in the brain and in the liver region. This is in contradiction with the concept that metabolic activity of tissues should determine the PCA reduction. Although we did not measure brain and liver metabolic activity, the depth of barbiturate anesthesia and eventually hypotermia associated with barbiturate anesthesia might have drastically reduced the oxygen consumption in both organs.

The anatomy of the observed region permitted an accurate measurement of brain kinetics only: brain parenchyma in fact was the main tissue component of the head region when spectra were collected. Liver tissue constituted only about 50% of the observed tissue when the active region of the EPR cavity was centered on the liver region. The PCA half life found by us is close to the value recently reported with the same label for the mouse liver by surface coil EPR experiments (3). We were unable to find consistent differences between the reduction rate in normoxia and moderate hypoxia. According to recent data (11), the *in vitro* doxylstearate reduction rate increases moderately when the physiological tissue oxygen concentration was

decreased by 10%. Similar data are not available for PCA and we do not know the intracellular oxygen tension we obtained during 10% O_2 -90% N_2 ventilation. In addition, mice were not artificially ventilated and could hyperventilate in response to the hypoxia challenge. Although the mouse acute intravenous toxicity data indicate a LD50 at 15 mM/kg for PCA (14), quite far from the PCA dosage employed in this study, our anesthetized and restrained animals could not tolerate more severe hypoxia. This might provide a faster reduction rate when compared to breathing air (Fig. 2).

The measurement of oxygen availability in intact tissue has proven to be full of technical difficulties. Although *in vivo* EPR spectroscopy is still in an early phase, EPR spectroscopy and/or imaging are very sophisticated technologies which in the future may provide new information about the basis of human disease and pathology. The major long-term benefits may come from their contribution to the basic understanding of the biochemical role of oxygen and its free radicals in the pathophysiology of disease.

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