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RAPID FREE RADICAL REDUCTION IN THE PERFUSED RAT LIVER

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The reduction of nitroxide free radicals was investigated in detail by Electron Paramagnetic Resonance (EPR) spectroscopy in perfused liver. The nitroxide free radical was rapidly reduced to the corresponding hydroxylamine more efficiently at the lower flow rate of 8 [ml/min], while at higher flow rates, the amount of reduced nitroxide showed a significant decrease. Oxidation of hydroxylamine using hydrogen peroxide provided dynamic information concerning the reduction of the free radical within the liver. In addition, liver homogenates were also investigated to determine the level of nitroxide uptake. The results suggested that a portion of the infused nitroxide was taken up by the liver and cleared from the circulation.

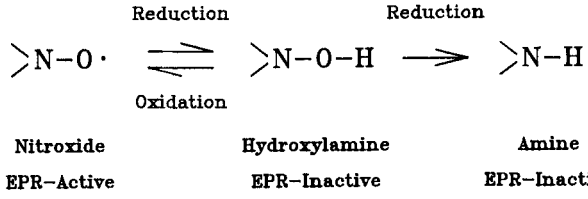
KEY WORDS: Liver, perfusion, free radical, reduction, EPR.

Abbreviations EPR, electron paramagnetic resonance; G, gauss; GC, gas chromatography; GHz, giga hertz; M, moles per liter; R.P.M., revolutions per minutes; TEMPOL, 4-hydroxy-2,2,6,6-tetramethyl-1-piperidinyloxy; W, watts.

INTRODUCTION

Paramagnetic nitroxides have been used as imaging agents for electron spin resonance (ESR) studies[1–3] and as contrast agents for magnetic resonance imaging (MRI)[4,5] in animals. These studies have indicated that the signal intensities decrease with cumulative time. The reduction of nitroxides in tissue homogenates has demonstrated that this process occurs *in vivo*.[4–6] For instance, the nitroxyl moiety is reduced to the hydroxylamine, which is a non-paramagnetic species (all spins are paired). The electron paramagnetic resonance (EPR)-inactive hydroxylamine is oxidized to its corresponding nitroxide by hydrogen peroxide,[5,7] which can be further reduced to the amine under specific conditions as follows:

Scheme I



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However, in previous studies, the tissue homogenates did not provide information concerning a specific organ regarding the reduction. Thus, we have performed liver perfusion experiments, which differ from studies using tissue homogenates in this regard. The liver is considered to be the organ most responsible for nitroxide reduction *in vivo*. Perfused liver is a useful model system for investigation of this reaction, but has not yet been used to investigate the reduction of a stable free radical as a function of the whole organ.

In the present report, we describe the dynamic aspects of stable free radical reduction in perfused rat liver. Not only various concentrations of the nitroxide spin label but also different flow rates of the nitroxide solution were examined. The amount of oxidized hydroxylamine (nitroxide radical) were measured by EPR spectroscopic techniques as a function of perfusion time. In addition, rat liver homogenates were investigated to determine the level of nitroxide free radical taken up by the liver at the end of the perfusion experiment.

EXPERIMENTAL PROCEDURES

Perfusion of Liver. Male Wistar rats (13 weeks old) weighing 300–350 g were used in these experiments. Each animal was anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally) and the abdominal wall was opened. A modified Krebs-Henseleit bicarbonate hemoglobin-free buffer consisting of 118 mM NaCl, 24.9 mM $NaHCO_3$, 0.60 mM KH_2PO_4, 2.40 mM K_2HPO_4, 2.57 mM $MgCl_2$, 1.27 mM $CaCl_2$, and 10 mM glucose (pH 7.4) saturated with 95% oxygen and 5% carbon dioxide was used as the perfusate.[8,9] The perfusate was maintained at 37°C in a water bath and the gas mixture was continuously passed through it. The buffer solution was pumped into the liver via a cannula placed in the portal vein. To ensure the appropriate delivery of perfusate to the liver, the small branch of the portal vein connected to the stomach was tied with a cotton string.[10] After purging of the blood from the liver with blank buffer, the perfusate was switched to the buffer solution containing 20 μM 4-hydroxy-2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPOL, Aldrich Chemical Co., Milwaukee, USA). Effluent from the second cannula placed in the vena cava through the right atrium was collected as shown in Figure 1. At the beginning of surgery, the flow rate was kept moderately low

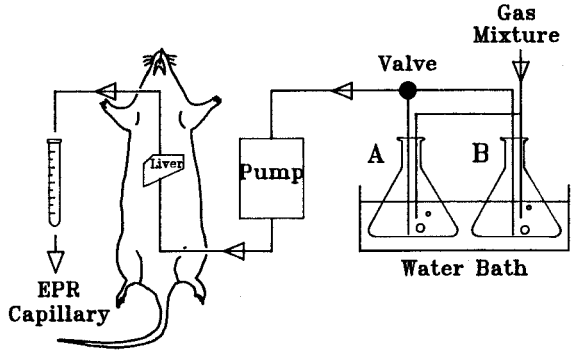

FIGURE 1 Schematic representation of the experimental setup for rat liver perfusion. *A* is the buffer solution containing 20 μM TEMPOL. *B* is the blank buffer solution used to purge the liver blood.

until the effluent cannula was inserted and secured, following which it was increased. The flow rate was determined based on the volume coming out of the liver, and the perfusate was not recirculated.

After switching to the perfusate containing TEMPOL, the effluent from the liver was collected approximately every 5 minutes until the end of the experiment. Each effluent fraction was sampled for EPR measurements. The perfusion experiment was stopped after approximately one hour. Leakage of the perfusate during the experiment was found to represent only a few percent.

Reduced nitroxide was oxidized by the addition of an equal volume of 30% hydrogen peroxide to the effluent from the liver.[5] The mixture was vortexed from time to time at ambient temperature. EPR measurements of the reoxidized sample solutions were obtained after about 48 hours.

In addition, GC (Hewlett Packard model 5890A) was also performed with a 30 m, 0.25 mm i.d. of DURABOND-1 (film thickness 0.25 μm) capillary to determine the existence of further reduced hydroxylamine in the effluents. The amine was confirmed by the known concentration of 2,2,6,6-tetramethyl-4-piperidinol (Tokyo Chemical Co.).

Liver homogenates. To examine further trace of the nitroxide radical and/or the hydroxylamine taken up by the liver, we homogenized the liver after purging with the blank buffer solution for several minutes. The liver was immediately removed from the rat, weighed, and homogenized by ten strokes at ~5200 R.P.M. in a rotating Teflon pestle in a glass mortar (Iuchi Digital Homogenizer, Japan) 1/3:w/v in 0.1 M phosphate buffer at pH 7.4. The homogenate was centrifuged at 3200 R.P.M. for 20 minutes and the supernatant was separated. Then, equal volumes of 30% H_2O_2 were added to aliquots of the supernatant solution to oxidize hydroxylamine to the nitroxide. EPR measurements were then taken to detect the amount of nitroxide in the liver.

EPR measurements. The time course of nitroxide reduction was measured by a JEOL RE3X X-band EPR spectrometer at room temperature. This spectrometer was equipped with a cylindrical TE_{011} mode cavity with MnO as a standard. We used one ($g = 2.034$) of the MnO signals to calibrate the nitroxide $m_I = +1$ transition intensity for each measurement. The first derivative peak-to-peak EPR signal intensity was compared. EPR conditions were as follows: microwave frequency, 9.04 GHz; microwave power, 10 mW; modulation amplitude, 1.0 G; time constant, 0.1 second; and scan rate, 50 G per minute. These values were maintained throughout the measurements.

RESULTS AND DISCUSSION

The hemoglobin-free perfused rat liver is a convenient model in which to study the dynamic aspects of nitroxide reduction. We chose TEMPOL as a water-soluble nitroxide free radical. Various concentrations and flow rates of the nitroxide free radical were examined to evaluate reduction of the free radical in the perfused liver. Based on different concentrations and flow rates in the liver perfusion experiments, we found that 20 μM and ~8 [ml/min], are adequate conditions to verify the reduction of nitroxide in the system. At higher concentrations (for instance, 100 μM) it is difficult to determine the reduction of nitroxide due to the small changes in signal.

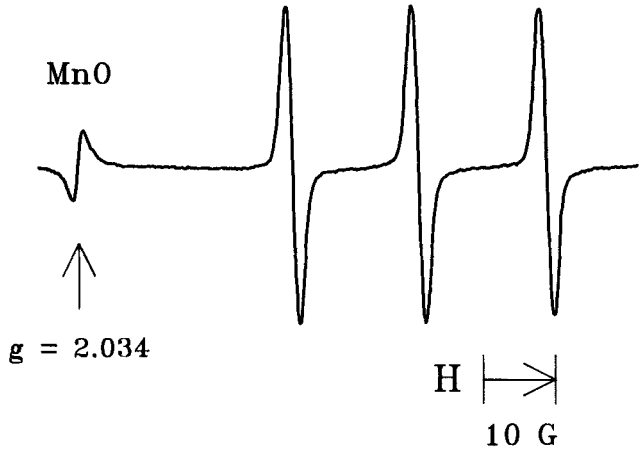

FIGURE 2 EPR spectrum of the original buffer solution in the presence of TEMPOL, and the MnO standard signal (g = 2.034). The nitrogen transition ($m_I = +1$) was divided by the standard signal to normalize the observed spectra. See text for details concerning EPR conditions.

Perfusion of the Liver. The original perfusate containing TEMPOL was measured to determine the initial EPR signal intensity prior to the experiment (Figure 2). To normalize each EPR measurement, the nitroxide signal ($m_I = +1$) of the nitrogen triplet was divided by the Mn^{2+} standard signal intensity (g = 2.034). At the end of the measurements, the ratios of normalized EPR intensity were plotted against the perfusion time. EPR measurements of the each effluent fraction were carried out at approximately 5 minute intervals. The each EPR intensity did not change during the time course of measurements and stayed the same for at least 20 minutes.

At the beginning of the perfusion experiments, TEMPOL nitrogen intensities were very weak and did not change significantly as a function of time as shown in Figure 3. Strikingly, most of the nitroxide was reduced by the rat liver in a single circulation. The normalized EPR intensities remained at a similar level during the 60 minutes of perfusion.

Figure 4 shows EPR spectra of the nitroxide (hydroxylamine oxidized by hydrogen peroxide) at the corresponding effluent times. We were able to observe the dynamic aspects of nitroxide reduction in the perfused liver as a function of time. At the beginning of the experiment, the nitroxide nitrogen triplet signal was very weak. The signal began to grow in intensity for about 5 minutes, after which it reached a steady state level which was maintained throughout the course of the experiment.

The EPR intensities obtained take into account the dilution of nitroxide by the addition of H_2O_2, and the results are presented as normalized intensities in Figure 5. At the beginning of the experiment, the reoxidized nitroxide had an EPR intensity of nearly zero, and approximately 20% of the nitroxide was obtained within the first 5 minutes of perfusion. It is obvious that the nitroxide is diluted in the liver lobule and begins to be reduced more effectively in the liver system. These factors were reflected in the EPR signal intensities. After about 10 minutes,

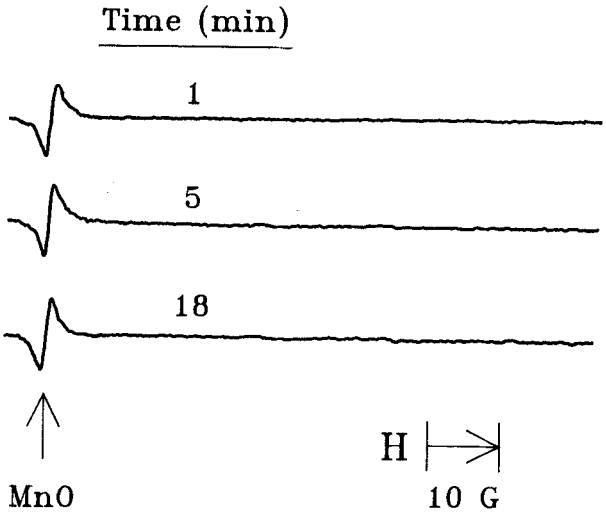

FIGURE 3 Representative EPR spectra of the perfusate collected at 1, 5, and 18 minutes during liver perfusion. The left side peaks represent the standard MnO signal. The *g*-value of MnO is 2.034.

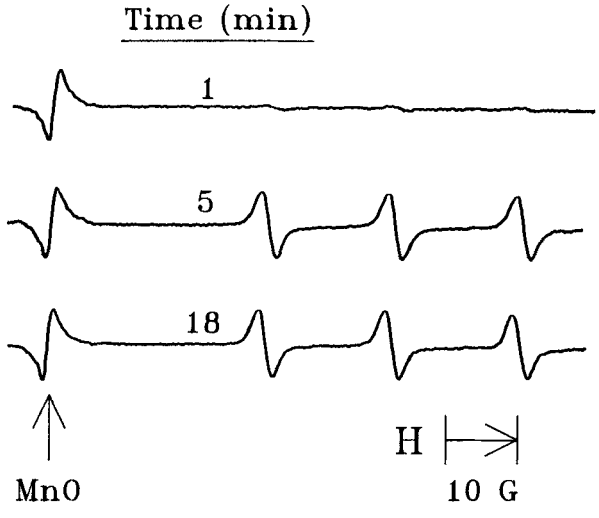

FIGURE 4 EPR spectra of the restored nitroxide. Each collected effluent time corresponds to each of the previous spectra of Figure 3. Note that the effluent volume was doubled by the addition of H_2O_2.

EPR intensities reached a maximum and gradually deceased as presented in Figure 5, but the normalized EPR intensities did not change much toward the end of the perfusion. It is noteworthy that less than half of the original nitroxide radical level was restored by the addition of H_2O_2. Although hydrogen peroxide may not be the best oxidizing agent to complete this reaction, we used it to restore the hydroxylamine.

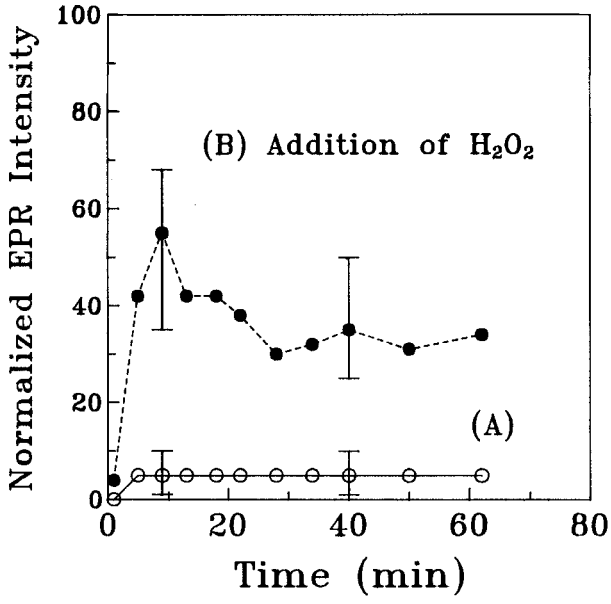

FIGURE 5 Normalized EPR intensities versus the effluent time. (A) shows normalized EPR intensities of the effluent from the rat liver. (B) shows the normalized EPR intensities after the oxidation of (A).

There are three important points to note in this liver perfusion experiment. Firstly, a portion of the nitroxide passes through the liver with no change, and we found that the amount of unchanged nitroxide in the effluent was increased at higher flow rates. Secondly, a further portion is reduced to hydroxylamine during the passage and this hydroxylamine was oxidized to the nitroxide by hydrogen peroxide. Changing the flow rate from 8 to 14 [ml/min] causes a drop in the level of reduction by about 25%. The results at various flow rates indicated that a flow rate of *ca.* 3 ml per gram of liver, which supplies adequate oxygen,[11] decreases reduction of the nitroxide further. Thirdly, the infused nitroxide may be changed to other diamagnetic species during its passage through the liver, and these species can not be detected by the present method. In addition, some of the infused nitroxide is taken up by the liver. Thus, in an attempt to follow the nitroxide we used the liver homogenates and capillary GC measurements.

Further Traces. We used liver tissue homogenates to determine whether some TEMPOL was taken up by the liver. Switching back to the blank buffer solution for several minutes, the liver was isolated and homogenized. No EPR signal was detected in either the purged buffer solution or the homogenates. With the addition of H_2O_2, approximately 10% of the nitroxide signal was measured by EPR spectroscopy. Thus, 10% of the nitroxide was found in the liver. Although the missing nitroxide has been recovered by this additional experiments, other diamagnetic species such as amines in the effluent are not easily identified.

However, we used capillary GC to measure the levels of reduced nitroxide (amine). The starting concentration of TEMPOL (20 μM) was too low to detect

using any analytical methods. The amine compound in the 300 ml of effluent obtained at a flow rate of 8 [ml/min] was extracted with chloroform and then the solvent was evaporated. Comparison of GC areas with the known concentration of 2,2,6,6-tetramethyl-4-piperidinol suggested that approximately 0.2% of the original TEMPOL was reduced to the amine in a single passage.

The present results have provided new insights concerning organ liver reduction of free radicals during circulation, and differed from previous results reported in studies of lung, liver and other homogenates including lung cells which indicated similar nitroxide reduction as a function of time.[3,12] Nitroxide reduction was dependent on the molecular structure of the nitroxide and exhibited mostly exponential decay. Moreover, no dynamic liver reduction of the spin label correlating with hydroxylamine and amine was observed by *in vivo* ESR.[1-3]

CONCLUSIONS

Our present results provide information regarding the detailed reduction of the stable nitroxide radical interacting with the organ liver. Even though the precise mechanisms of nitroxide reduction are still unknown, our findings are useful at least in three respects. Firstly, the liver steadily reduces ~95% of the 20 μM nitroxide free radical in a single passage, and the level of reduction is the same during perfusion for 60 minutes. Secondly, the liver reduces the stable free radical more effectively to hydroxylamine at lower flow rates; increasing the flow rate from 8 to 14 [ml/min] decreased the amount of hydroxylamine production by approximately 25%. Our results indicate that nitroxide reduction by the liver is strongly dependent on the flow rate. Moreover, approximately 0.2% of TEMPOL was reduced to the amine at 8 [ml/min]. However, the present results do not show the specific reduction site in the liver. Couet *et al.* suggested that sulfhydryl group instead of endogenous ascorbic acid is mostly responsible for the reduction in the liver.[4] Thirdly, liver homogenates showed that a portion of the infused nitroxide was taken up by the liver and consequently cleared from the circulation.

Acknowledgements

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