Analysis of Nitroxyl Spin Probes in Mouse Brain by X-Band ESR with Microdialysis Technique

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Received 18 June 2007; revised 9 October 2007; accepted 17 October 2007

Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jps.21258

ABSTRACT: Stable nitroxyl radicals are widely used in electron spin resonance (ESR) studies *in vivo* to determine ROS generation, but there are insufficient data on how their distribution to various tissues, excretion, and/or systemic signal decay affect the signal decay in a region of interest. Here, we evaluated the level of spin probe in the brain using a microdialysis combined with X-band ESR spectroscopy, to clarify the BBB permeability of different spin probes. We also determined the association between PROXYL spin probe signal decay in the head and the probe's level in the brain, its excretion in urine, and its rate of signal decay in other areas and tissues. Dialysate recovered from the mouse prefrontal cortex was used to determine the total spin probe level in the brain by X-band ESR spectroscopy. There was a positive correlation between the level of spin probes in the brain and their partition coefficients. Furthermore, the *in vivo* decay rate of the nitroxyl radical signal in the head was associated with the probes' level in the brain, but not with its systemic signal decay rate or excretion into urine. These basic data may support the use of PROXYLs as site-specific ROS probes in the brain. © 2008 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 97:4101–4107, 2008

Keywords: nitroxyl radical; microdialysis; electron spin resonance; blood–brain barrier; free radical; pharmacokinetics

INTRODUCTION

Many studies have shown that free radicals and reactive oxygen species (ROS) cause lipid peroxidation,[1] protein oxidation, and oxidative damage to DNA,[2] all of which damage cells. Free radicals and ROS are important mediators of brain injury, such as that caused by ischemia reperfusion.[3,4] Thus, the noninvasive and direct evaluation of ROS generation *in vivo* is important for understanding the pathogenic mechanisms of oxidative stress in brain injury.

In the last few decades, the development of *in vivo* electron spin resonance (ESR) spectroscopy[5–9] and *in vivo* ESR imaging[10–16] has made it

Abbreviations: BBB, blood–brain barrier; carbamoyl-PROXYL, 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-l-oxyl; carboxy-PROXYL, 3-carboxy-2,2,5,5-tetramethylpyrrolidine-l-oxyl; carboxy-TEMPO, 4-carboxy-2,2,6,6-tetramethylpiperidine-1-oxyl; ESR, electron spin resonance; hydroxyl-TEMPO, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; methoxycarbonyl-PROXYL, 3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine-l-oxyl; MW, molecular weight; oxo-TEMPO, 4-oxo-2,2,6,6-tetramethylpiperidine-1-oxyl; Po/w, partition coefficients between *n*-octanol and PBS; ROS, reactive oxygen species.

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Journal of Pharmaceutical Sciences, Vol. 97, 4101–4107 (2008)

possible to measure paramagnetic species in living animals noninvasively. One kind of paramagnetic species, stable nitroxyl radicals, has been widely used as spin probes for *in vivo* ESR to evaluate ROS generation. When a spin probe is administered to mice or rats, the signal decay rates of the nitroxyl radical are enhanced by ROS during oxidative stress.[17–19] The enhanced signal decay rate could be used as an index of ROS generation, because the administration ofantioxidants suppresses the enhanced signal decay.[3,19] However, some difficulties remain in the interpretation of results from this *in vivo* technique, because signals measured in a region of interest could reflect the overall level of the probe in the circulatory system, rather than local ROS generation. Because a steady-state signal decay represents several factors, including distribution of the probe from the blood into the tissues,[20,21] signal reduction caused by enzymatic reactions[22,23] or reducing agents[24] in the tissues, and excretion of the probe,[20,25] these factors might also influence the signal decay recorded at the region of interest. Currently, there are insufficient data to dissect the contributions of these various factors to the signal obtained from a specific region of the body.

The endothelial cells forming the blood–brain barrier (BBB), unlike the vascular endothelial cells elsewhere, are characterized by tight intercellular junctions that limit drug penetration into the brain parenchyma. In addition, the BBB expresses various efflux transporters, such as P-glycoprotein,[26] and organic anion transporting polypeptides[27] that can remove agents that cross the BBB. Therefore, the efficiency of the distribution of spin probes into brain must contribute substantially to the measurable level of signal decay in the brain. The efficiency of spin probe distribution into the brain in living animals has been evaluated by imaging analysis.[10,14] ESR imaging or the autoradiography have clarified that 3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine-l-oxyl (methoxycarbonyl-PROXYL) has good BBB permeability. However, although the imaging analysis is useful, it is hard to obtain an accurate measure of spin probes that have weak BBB permeability. A quantitative approach to measuring ROS levels requires that the level of spin probe in the region of interest be known. Intracerebral microdialysis allows the direct sampling of brain interstitial fluid via a dialysis fiber implanted into a specific area of the brain. The major advantage of this technique is that it provides pharmacokinetic profiles of compounds in the brain of the same animal at different time points. We therefore combined the microdialysis technique with X-band ESR to obtain data on spin probe levels in the brain.

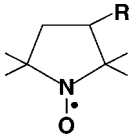
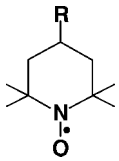
In the present study, we used this combined technique to clarify the efficiency of spin-probe permeability into the brain. Furthermore, we examined whether the signal decay of PROXYL spin probes detected at the head region was related to the efficiency of their distribution to brain, their excretion in urine, and/or to the signal decay in other regions of the body.

EXPERIMENTAL SECTION

Chemicals

Table 1 lists the nitroxyl radicals used in this study. 4-carboxy-2,2,6,6-tetramethylpiperidine-1-oxyl (carboxy-TEMPO), 4-oxo-2,2,6,6-tetramethyl-

Table 1. Structures, Abbreviations and *n*-Octanol/Water Partition Coefficients (Po/w) of Nitroxyl Radicals

Ring Structure	Substituent (*R*)	Abbreviation	Po/w
	–COOH	Carboxy-PROXYL	0.02 ± 0
	$-CONH_2$	Carbamoyl-PROXYL	0.58 ± 0.04
	$-COOCH_3$	Methoxycarbonyl-PROXYL	9.50 ± 0.11
	–COOH	Carboxy-TEMPO	0.04 ± 0
	=O	Oxo-TEMPO	1.59 ± 0.20
	–OH	Hydroxy-TEMPO	2.68 ± 0.05

Each value represents the mean ± SEM. Each experiment was repeated three times.