Free Rad. Res., Vol. 23, No. 1, pp. 27–32
Reprints available directly from the publisher
Photocopying permitted by license only

DETECTION OF FREE RADICALS BY MICRODIALYSIS/SPIN TRAPPING EPR FOLLOWING FOCAL CEREBRAL ISCHEMIA-REPERFUSION AND A CAUTIONARY NOTE ON THE STABILITY OF 5,5-DIMETHYL-1-PYRROLINE N-OXIDE (DMPO)

LAURA L. DUGAN*, TIEN-SUNG LIN[a]*, Y.-Y. HE, CHUNG. Y. HSU and DENNIS W. CHOI

Department of Neurology, School of Medicine, Washington University, St. Louis, Missouri 63110, and [a]Department of Chemistry, Washington University, St. Louis, Missouri, 63130, USA

(Received January 21, 1994; revised received April 28, 1994)

We have examined free radical production in a rat model of focal cerebral ischemia using microdialysis coupled with EPR analysis. A microdialysis probe was inserted 2 mm into the cerebral cortex, supplied by the right middle cerebral artery (MCA), and after a 2-hour washout period with artificial cerebral spinal fluid (ACSF), the perfusate solution was changed to ACSF containing the spin trapping agent, 5,5-dimethyl-1-pyrroline N-oxide (DMPO). No free radicals were detected by DMPO during the pre-ischemia period. Both common carotid arteries and the right MCA were then ligated for 90 minutes. Microdialysate collected every 15 min during the ischemic period demonstrated predominantly superoxide or peroxyl radical production. After release of the occlusive sutures, hydroxyl radical became apparent initially, then thiyl and carbon centered radicals appeared later in samples collected every 15 min for two hours following cortical reperfusion. Careful studies on the purification and stability of DMPO solution were performed to circumvent artifacts and spurious signals.

KEY WORDS: Superoxide, hydroxyl radicals, EPR, spin trapping, microdialysis, ischemia.

INTRODUCTION

There is a growing consensus that free radical injury may play a key role in neuronal cell death following ischemic or traumatic insults to the central nervous system (CNS).[1-3] Ischemia induced pathologic processes may be responsible for the single greatest cause of death in this country – heart disease and stroke. The cellular or tissue injury may occur not during the period of hypoxia but rather during reperfusion when oxygen is reintroduced to the tissue.[4] It has been suggested that biochemical reactions can produce excess oxygen free radicals upon reperfusion, and that these radicals can contribute to tissue injury during ischemia and reperfusion.

We have employed EPR spin trapping techniques to monitor free radical produc-

*To whom correspondence should be addressed.

Paper presented at the 4th International Symposium on Spin Trapping and Organic EPR Spectroscopy, Oklahoma City, USA, October 1993

tion in the ischemia-reperfusion process in a reversible rat middle cerebral artery occlusion (MCAO) model of focal ischemia.[5] A spin trapping agent, DMPO (5,5-dimethyl 1-pyrroline N-oxide), was used to trap the reactive and transient free radicals released from the cells as stable nitroxide adducts. Microdialysis allowed us to follow the production of free radicals continuously at different stages of ischemia and reperfusion processes.

In the course of our investigation, we encountered some difficulties in the purification and the stability of the trapping agent. DMPO yielded artifacts and spurious EPR signals which were assignable to the spin adducts of hydroxyl radicals and dimerized DMPO-nitroxide. Here we report our preliminary results of the microdialysis/EPR experiments, and some cautionary comments about the stability of DMPO solutions.

MATERIALS AND METHODS

Male Long Evans rats were used in the experiments. After anesthesia with pentobarbital, a microdialysis probe was inserted 2 mm into the cerebral cortex supplied by the right MCA, and the tubing was flushed for 2 hours with artificial cerebral spinal fluid (ACSF) at 5 μl/min. The dialysis solution was changed to ACSF containing the spin trapping agent, DMPO (500 mM) and a chelating agent, diethylenetriaminepentaacetic acid (DETAPAC, 100 μM) which was prepared immediately prior to use. Ischemia was induced by ligation of both common carotid arteries and the right MCA for 90 min. Microdialysate samples were collected every 15 min during pre-ischemia (1 hour), intra-ischemia (90 min), and reperfusion (120 min). EPR spectra were taken immediately after the sample collections.

The spin trapping agent, DMPO, was purchased from Sigma Chemical Co. (St. Louis, MO) and was purified by multiple treatments with activated charcoal until it became colorless. We also purified DMPO by vacuum sublimation (10^{-5} torr). The sublimed DMPO is colorless crystal with a m.p. of 25°C. However, we noticed that when DMPO was slightly heated to ~150°C in the presence of air, it melts and remains as liquid even at room temperature. It has been reported that DMPO related pyrroline may dimerize or polymerize[6] upon heating and therefore lowered the m.p. The dimerized or polymerized DMPO is an oily brownish liquid.

A Bruker ER200D EPR spectrometer (X-band) was employed in the experiments. The sample was loaded into a flat quartz cell (100 μl). The microwave power was kept at about 3–5 mW level. The modulation amplitude was set at 2 G.

RESULTS AND DISCUSSION

(1) *EPR Spectra of DMPO Solutions*

As in control experiments, we checked EPR signals of the DMPO solutions during the course of microdialysis, which often lasted 5 to 6 hours. From time to time, we observed some artifacts and spurious EPR signals. To check the stability of DMPO, we took EPR spectra of concentrated 2M DMPO solutions (containing DETAPAC chelating agent) as a function of time and temperature. The solution was kept in a plastic syringe, in the dark and at room temperature or at a slightly elevated temperature to emulate our microdialysis experiments.

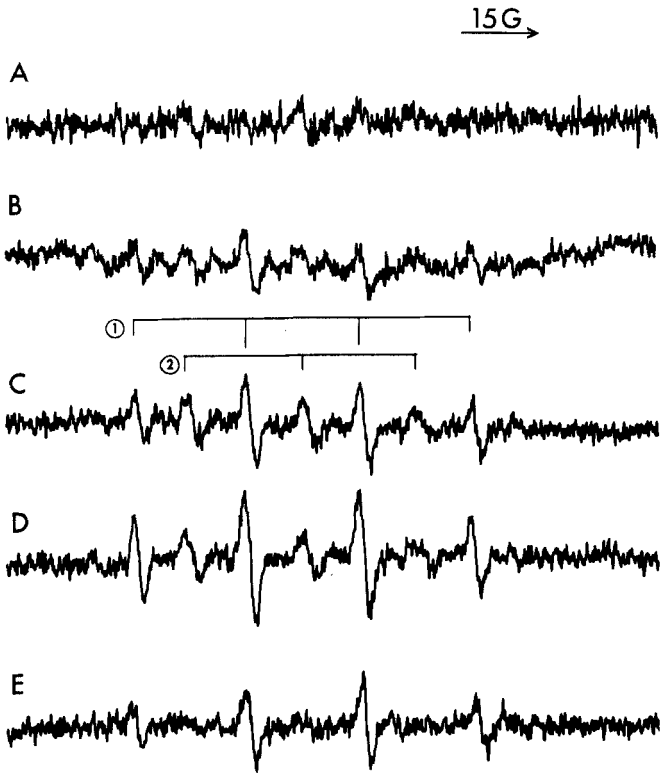

FIGURE 1 Room temperature EPR spectra of 2M DMPO solution (except spectrum E) containing 100 μM DETAPAC as a function of standing time and temperature: (A) freshly prepared 2M DMPO solution. (B) 5 hours standing in a plastic syringe at room temperature. (C) 24 hours at room temperature. (D) 24 hours at room temperature and heated to 50°C for 3 min. (E) 0.2M DMPO containing 100 μM DETAPAC at 37°C for 3 hours. Assignments: spectral set 1 = DMPO-OH* 2 = unknown nitroxide.

The spectra were displayed in Figure 1. The results are summarized as follows:

(a) Freshly prepared sample: no free radical signal (Spectrum 1A).
(b) 5 hours: weak DMPO-OH· signal began to appear (Spectrum 1B).
(c) 24 hours: DMPO/OH· signal intensity increased, and another triplet (unknown nitroxide which may arise from "syringe nitroxide"[7]) appeared (Spectrum 1C).
(d) Brief warm-up to 50°C: To check the thermal stability, the solution from (c) was warmed up to 50°C for 3 minutes in the flat EPR cell. We observed a 2-fold increase of overall intensity (Spectrum 1D). The spectral intensity then remained the same for several days.
(e) The temperature effect is further supported in a different experiment: substantial DMPO-OH· signal was observed in a 0.2M DMPO containing DETAPAC solution which was kept at 37° for 3 hours (Spectrum 1E). No signal was observed in a freshly prepared DMPO solution under the same conditions.

Thus several hours of exposing the DMPO solution in the air even at room temperature can yield spurious EPR signals. The OH· signal may arise from the reac-

tion of trace amount of iron ions and dissolved oxygen in the solution or hydrolysis process[8] even though we took precautions to remove the iron by the chelating agent.

(2) *EPR Spectra of Microdialysate*

The EPR spectra of microdialysate containing DMPO and DETAPAC during focal cerebral ischemia and reperfusion in the right MCA cortex were shown in Figure 2. Note that the concentration of DMPO used in the microdialysis experiment was 4 times less than in controls. The background spurious signal should be much less than that displayed in Figure 1. The results are summarized as follows:

(a) Pre-ischemia period: no detectable free radical signal is observed (Spectrum 2A).
(b) Intra-ischemia period: detectable spin adduct signal is present (Spectrum 2B).
(c) Reperfusion 0–15 min: hydroxyl radicals are generated; a 6-peak spectrum consistent with thiyl radicals[9] (a_N = 15.4 and a_H = 16.2 G); other radicals, such as lipid peroxidation products, may also be present (Spectrum 2C).
(d) Reperfusion 75–90 min: OH· is still detected, plus the same components as in 2C (Spectrum 2D).

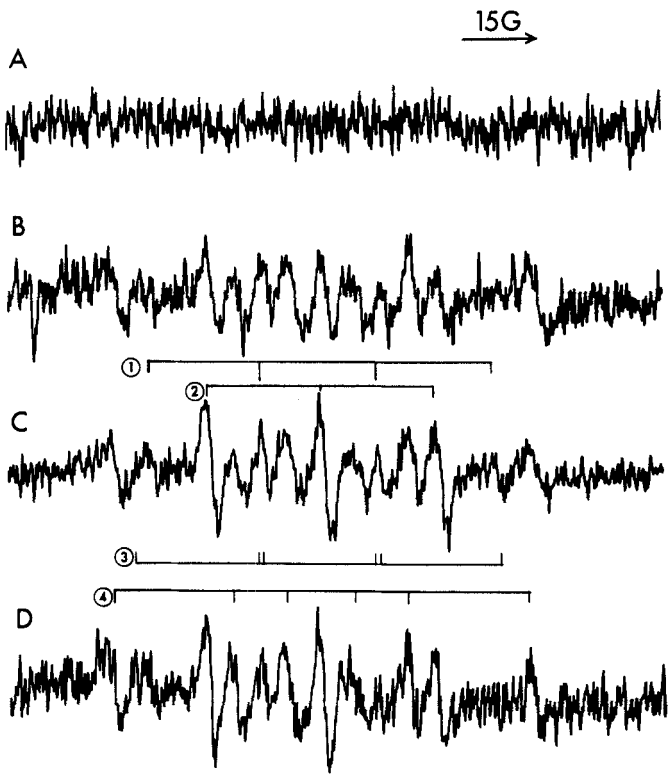

FIGURE 2 EPR spectra of microdialysate using DMPO trapping agent from rat cortex during middle cerebral artery occlusion (MCAO) and reperfusion: (A) pre-ischemia period, (B) intra-ischemia, (C) reperfusion 0–15 min, (D) reperfusion 75–90 min. Assignments: spectral set 1 and 2 are the same as Figure 1, 3 = thiyl radical adducts 4 = carbon centered adducts.

Increased production of OH· at the onset of reperfusion was further confirmed using a salicylate assay experiment: products of OH· attack on salicylate, the 2,3- and 2,5-dihydroxybenzoic acids, were measured by HPLC. We observed a 30–50 fold increase in the production of OH· with reperfusion.

Our experimental procedure and the results differ from those of a previous microdialysis study of cerebral ischemia by Zini *et al.*[10] in four respects: (1) we used a reversible MCAO model, which is designed to mimic a focal ischemic insult (stroke); this contrasts with 4-vessel occlusion model used by Zini, which produces global ischemia; (2) we placed our probe in the parietal cortex, as opposed to an intrastriatal placement in the Zini study; (3) we detected hydroxyl, thiyl and lipid radical adducts with reperfusion; in the Zini study, hydroxyl and lipid peroxide adducts only were seen; (4) the Zini study used 4-POBN (4-pyridyl-N-oxide *t*-butyl nitrone) as the effective trapping agent.

Superoxide radicals are formed by one electron reduction of molecular oxygen. In biological processes, $O_2^{-\cdot}$ can be formed when xanthine oxidase acts to oxidize xanthine (degraded from the energy carrier ATP) or other substrates. Other sources of free radicals during ischemia-reperfusion may include metabolism of arachidonic acid by cyclo-oxygenase and lipoxygenase, generation of oxygen radicals by mitochondria, and production of radical species by P450 enzymes and oxidases. $O_2^{-\cdot}$ is converted to OH· radical by the iron catalyzed Haber-Weiss reaction. Antioxidants such as glutathione, ascorbate and α-tocopherol which provide important defenses during oxidative stress may also be oxidized. The oxidation of glutathione may produce detectable radical adducts.

CONCLUSION

Microdialysis is a viable technique to detect changes in the biochemical profile during focal cerebral ischemia-reperfusion. The technique allows us to monitor the production of free radicals *in vivo*. We have taken precautions to minimize the surgical damage to the brain in microdialysis studies.

The progression of free radical formation in the ischemia – reperfusion processes are: Pre-ischemia: no free radical formation. Intra-ischemia: superoxide or peroxyl radicals are formed. Reperfusion: hydroxyl radicals are initially formed, with thiyl radicals becoming apparent at the same time. Thus, our findings are consistent with the implications of previous work on this model that ischemic brain injury may be attributed at least in part to the generation of excess amounts of free radicals.[5] Careful determination of the temporal and spatial profile of different radical species generated during ischemia-reperfusion may broaden our insight into the sources of radical production, and the future development of therapeutics directed at free radical mediated mechanism.

DMPO is an effective spin trapping agent which allows one to detect reactive radicals, such as superoxide and hydroxyl radicals, formed in the neuronal injury resulting from acute brain insults and damage. However, the DMPO solution itself could give rise to artifacts and spurious signals if it is not freshly prepared: 5 hours standing at room temperature will produce a detectable DMPO-OH· signal. Greater free radical intensity was observed after long standing time and at higher temperature.

Acknowledgements

This work was supported in part by a grant from the CSNSI (Center for the Study of Nervous System Injury), Washington University Medical Center. CYH and YYH are supported by NS28995 and NS25545. TSL are supported by NSF (CHE-9106499).

References

1. E.S. Flamm, H.B. Demopoulos, M.L. Seligman, R.G. Roser and J. Ranserhoff (1978) Free radical in cerebral ischemia. *Stroke*, **9**, 445–447.
2. W. Cao, J.M. Carney, A. Duchon, R.A. Floyd and M. Chevion (1988) Oxygen free radical involvement in ischemia and reperfusion injury to brain. *Neuroscience Letters*, **88**, 233–238.
3. J.M. Braughler and E.D. Hall (1989) Central nervous system trauma and stroke. I. Biochemical considerations for oxygen radical formation and lipid peroxidation. *Free Radical Biology & Medicine*, **6**, 289.
4. B. Halliwell (1991) Reactive oxygen species in living system: source, biochemistry, and role in human disease. *American Journal of Medicine*, **91**, 14S–22S.
5. Y.Y. He, C.Y. Hsu, A.M. Ezrin and M.S. Miller (1993) Polyethylene glycol-conjugated superoxide dismutase in focal cerebral ischemia-reperfusion. *American Journal of Physiology*, **265**, H252–H256.
6. R.F.C. Brown, V.M. Clark, M. Lamchen and S.A. Todd (1959) Experiments towards the synthesis of Corrins. Part VI. The Dimerization of pyrroline 1-oxide to 2-(1′-hydroxypyrrolidin-2′-yl)-pyrroline 1-oxide. *Journal of the Chemical Society*, **100**, 2923–2925.
7. G.R. Buettner, B.G. Scott, R.E. Kerber and A. Mugge (1991) Free radicals from plastic syringe, *Free Radical Biology and Medicine*, **11**, 69–70; G.R. Buettner and M.K. Sharma (1993) The syringe nitroxide free radical – part II, *Free Radical Research Communication*, **19**, S227–S230.
8. M.J. Burkitt and R.P. Mason (1991) Direct evidence for *in vivo* hydroxyl-radical in experimental iron overload: An EPR spin-trapping investigation, *Proceedings of National Academy of Sciences*, **88**, 8440–8444.
9. C.H. Kennedy, D.F. Church, G.W. Winston, W.A. Pryor (1992) Tert-butyl hydroperoxide-induced radical production in rat liver mitochondria. *Free Radical Biolog & Medicine*, **12**, 381–387.
10. I. Zini, A. Tomasi, A. Grimaldi, V. Vannini and L.F. Agnati (1992) The detection of free radicals during brain ischemia and reperfusion by spin trapping and microdialysis. *Neuroscience Letters*, **138**, 279–282.

Accepted by Professor Ed. Janzen