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ALPHA-PHENYL-TERT-BUTYL-NITRONE (PBN) ATTENUATES HYDROXYL RADICAL PRODUCTION DURING ISCHEMIA-REPERFUSION INJURY OF RAT BRAIN: AN EPR STUDY

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α-phenyl-tert-butyl-nitrone (PBN) a spin adduct forming agent is believed to have a protective action in ischemia-reperfusion injury of brain by forming adducts of oxygen free radicals including ·OH radical. Electron paramagnetic resonance (EPR) has been used to both detect and monitor the time course of oxygen free radical formation in the *in vivo* rat cerebral cortex. Cortical cups were placed over both cerebral hemispheres of methoxyflurane anesthetized rats prepared for four vessel occlusion-evoked cerebral ischemia. Prior to the onset of sample collection, both cups were perfused with artificial cerebrospinal fluid (aCSF) containing the spin trap agent α-(4-pyridyl-1-oxide)-N-tert butylnitrone (POBN 100 mM) for 20 min. In addition 50 mg/kg BW of POBN was administered intraperitoneally (IP) 20 min prior to ischemia in order to improve our ability to detect free radical adducts. Cup fluid was subsequently replaced every 15 min during ischemia and every 10 min during reperfusion with fresh POBN containing CSF and the collected cortical superfusates were analyzed for radical adducts by EPR spectroscopy. After a basal 10 min collection, cerebral ischemia was induced for 15 or 30 min (confirmed by EEG flattening) followed by a 90 min reperfusion. ·OH radical adducts (characterized by six line EPR spectra) were detected during ischemia and 90 min reperfusion. No adduct was detected in the basal sample or after 90 min of reperfusion. Similar results were obtained when diethylenetriaminepenta-acetic acid (100 μM; DETAPAC) a chelating agent was included in the artificial CSF. Systemic administration of PBN (100 mg/kg BW) produced a significant attenuation of radical adduct during reperfusion. A combination of systemic and topical PBN (100 mM) was required to suppress ·OH radical adduct formation during ischemia as well as reperfusion. PBN free radical adducts were detected in EPR spectra of the lipid extracts of PBN treated rat brains subjected to ischemia/reperfusion. Thus this study suggests that PBN's protective action in cerebral ischemia/reperfusion injury is related to its ability to prevent a cascade of free radical generation by forming spin adducts.

KEY WORDS: Cerebral ischemia, Reperfusion, Oxygen free radical, Hydroxyl radical, EPR, POBN, and PBN.

INTRODUCTION

Oxygen free radicals have been implicated in cerebral ischemia-reperfusion injury. Potentially the most important of the oxygen free radicals is hydroxyl radical (·OH). It is formed from O_2^- and H_2O_2 by the *Haber Weiss Reaction* or when H_2O_2 is utilized in the *Fenton reaction*. Iron released from the damaged cells is believed to catalyze the latter reaction.[1,2] The ·OH radical thus formed is highly reactive and

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capable of producing oxidative damage to almost all components of the living cell.[3] The ·OH radical is also believed to be capable of generating secondary oxygen free radicals. These, together with the O_2^- and H_2O_2 from which the ·OH radical was derived, are known to launch similar peroxidative attacks resulting in cell injury.[4]

Spin adduct forming agents have been widely used to detect oxygen free radicals by electron paramagnetic resonance (EPR) spectroscopy. Recently it has come to light that these agents trap the very radicals that they are used to detect.[5] PBN, one such spin trap agent has been shown to attenuate many of the effects of ischemia-reperfusion injury on brain.[6,7] It has been postulated that PBN mediates its beneficial effect by trapping free radicals including H_2O_2 radical.[6,8]

Spin trapping and EPR spectroscopy have been used to provide direct evidence of ·OH radical production in biological systems. Recently Zini *et al.* used α-(4-pyridyl-1-oxide)-N-tert butylnitrone (POBN), a spin trap agent, in the EPR detection of extracellular free radicals produced during brain ischemia in striatal microdialysate samples as carbon centered radical adducts.[9] In the present study POBN was used to directly detect and follow the release of ·OH radicals into cerebral cortical superfusates during ischemia-reperfusion of the rat brain. First we determined the period of ischemia that was necessary to produce a sustained release of ·OH radical. Then, in order to study the role of PBN in influencing ·OH radical production, we administered PBN systemically alone as well as in combination with topically administered PBN and similarly followed the EPR spectra in rats subjected to 30 min ischemia followed by reperfusion.

MATERIAL AND METHODS

Techniques of Cerebral Ischemia-reperfusion and Setting up Cortical Cup

Adult male Sprague-Dawley rats (350–450 gm) were anesthetized with halothane. After insertion of a tracheal cannula, anesthesia was maintained with a mixture of methoxyflurane in air. Body temperature was regulated at 37°C with an abdominal heating pad controlled by a rectal probe.

A femoral artery was cannulated to monitor mean arterial blood pressure (MABP) and for the withdrawal of arterial blood samples for pH and blood gas analysis. The right and left common carotid arteries were isolated and separated from their accompanying nerve trunks. A length of dental foss (Johnson & Johnson) was looped around each artery and exteriorized through the neck incision. The ends of each carotid snare were held together by adhesive tape. Following placement of the animals head in a Narashige SH-8 non-traumatic head holder, a continuous mid-line incision was made along the top of the skull and extending along the dorsum of the neck. After removal of the overlying muscles, the vertebral arteries were electrocoagulated with a monopolar electrode inserted into the alar foramina of the first cervical vertebra. The neck incision was then closed with Michel wound clips. The dorsal and dorsolateral surfaces of both cerebral hemispheres were exposed by removal of the overlying frontal and parietal bones, with a thin strip of bone left intact along the mid-line to protect the dorsal sagital venous sinus. The dura mater-arachnoid complex overlying both hemispheres was reflected, and oval cortical cups suspended in flexible mounting brackets were lowered onto both hemispheres, so that the frontal, parietal, and occipital cortical surfaces were exposed within the

cups. Each cup was filled with an artificial cerebrospinal fluid (aCSF) solution to ensure that there was no leakage of fluid from the cups.

The dorsal surface of the head was then covered with 4% agar in normal saline to protect the exposed surfaces of the skull and stabilize the cups. Monopolar EEG leads were placed in both cups with the tip of the electrode adjacent to the cortical surface. EEGs and arterial blood pressure were recorded on a Grass Polygraph. The cups were emptied and refilled with 310 μl of warmed (37°C), sterile aCSF (Na^+, 155.8 mEq/L; K^+, 2.95 mEq/L; Ca^{++}, 2.5 mEq/L; Mg^{++}, 1.85 mEq/L; Cl^-, 141.13 mEq/L; HCO_3^-, 22 mEq/L; Dextrose, 66.5 mg/dL; and Urea, 40.2 mg/dL) containing 100 mM POBN with or without 100 μM diethylenetriaminepenta-acetic acid (DETAPAC: added to prevent trace element catalyzed pseudo-adduct formation; Zini *et al.*, 1992), which had been bubbled with a gas mixture of 5% carbon dioxide in 95% nitrogen. The cup fluid was maintained at 37°C with a heat lamp and the cups were protected from light as POBN is photosensitive. In addition to adding POBN in the aCSF, it was also administered systemically (50 mg/kg, I.P.) 20 min prior to the onset of cerebral ischemia.

Cerebral ischemia was elicited by traction on the carotid snares and was maintained for 30 min. Successful induction of cerebral ischemia was evident from a rapid flattening of the EEG traces, which then remained isoelectric for the duration of ischemic episode. In the group of rats subjected to 15 min ischemia (n = 3), there was partial EEG recovery over the period of reperfusion.

Experimental Protocol

Four groups of animals were subjected to the 30 minute ischemia followed by a 90 minute reperfusion after the carotid snares had been cut and removed. The control ischemic group (n = 6) did not receive any PBN. The second group (n = 6) of animals received 100 mg/kg PBN IP administered 20 minutes prior to ischemia. A third group (n = 4) received 100 mM PBN topically administered in the artificial CSF, in addition to the 100 mg/Kg PBN IP. The fourth group (n = 8) of animals differed from the first only in that DETAPAC was present in the aCSF. In each of the four groups of animals CSF containing 100 mM POBN was sampled at similar intervals separately from cups covering each cortex. After a 20 min period for equilibration, during which the cup aCSF was replaced three times, a control basal superfusate sample was obtained after a 10 min collection period. Two ischemic samples were obtained after 15 min collection periods. Three reperfusion samples were obtained during the initial 30 min of reperfusion, each after a collection period of 10 min. Two more of such samples were obtained after two successive breaks of 20 min each thus covering a total of 90 min of reperfusion. The 310 μl of aCSF sample obtained from each cup was collected in microvials, immediately frozen in dry ice −20°C and maintained frozen until subsequent EPR study within 2-4 hrs.

Brain Lipid Extraction Procedure

In addition to the above protocol, a group of animals (n = 3) were administered 100 mg/kg PBN IP and 20 min later subjected to 30 min ischemia followed by 10 min of reperfusion as described above. At the end of reperfusion the animals were euthanized by decapitation, the brain removed and immediately frozen in liquid nitrogen. The frozen brain was ground and allowed to thaw in 10 ml of a spin trap solution composed of 10 mM PBN, 0.1 mM DETAPAC, and 0.1 M dimethylsulf-

oxide (DMSO). DMSO was included to stabilize the PBN-OH adduct. The above was added to 40 ml of 2:1 mixture of chloroform (Aldrich, catalog no. 31998-8) and methanol (Aldrich, catalog no. 27047-4), vortexed and centrifuged at 45,000 rpm for 15 min at 0°C The supernate chloroform-methanol mixture was transferred to separate test tube and dried with a stream of nitrogen. The dried extract was reconstituted with 1 ml of chloroform methanol. This sample was stored in ice (0-4°C) till subsequent EPR study.

EPR Analysis

All the samples were subjected to EPR analysis at room temperature in a flat quartz cell. The Varian E-109 EPR spectrometer settings for aCSF samples were as follows: microwave power 12.5 mW; time constant 0.5 s; scan range 100 G; scan time 8 min; gain for scan was 1.25×10^5; and microwave frequency of 9.0 GHz. The EPR signals thus obtained were used to calculate α_N, α_H^β, and peak to peak amplitude (cm). For samples obtained from cerebral lipid extraction the EPR settings were identical to those mentioned above except that a scan range of 200 G for a scan time of 16 min and a receiver gain of 5.0×10^4 was used. *Fenton's reaction*, started by adding 100 μM H_2O_2 and 1 mM ferrous ammonium sulfate to aCSF containing 100 mM POBN, was used as the standard for the analysis of ·OH radical generated EPR spectra. All chemicals used for the study were obtained from Sigma unless mentioned otherwise.

Statistical Methods: The mean and standard error of mean of the peak to peak amplitude of EPR signals obtained at each time interval were calculated. The results thus obtained were compared with those obtained in the control ischemia (30 min) animals using one-way analysis of variance (ANOVA) with 0.001 as the level of significance unless mentioned otherwise. The Bonferonni p value was used to determine the significance as multiple comparisons had to be made.

RESULTS

Essentially three types of EPR signals were obtained using POBN as a spin trap agent. The first one was a six line spectrum characteristic of the ·OH radical adduct of POBN. The hyperfine splitting constants were similar to the signal obtained from the *Fenton's reaction* (Figure 1), i.e., $\alpha_N = 15.4$ G and $\alpha_H^\beta = 2.5$ G. The signal form and values of splitting constants closely corroborate those previously reported for the ·OH radical adduct of POBN.[9,10] The second variety of EPR signal was consistent with the one produced by ascorbyl radical, i.e., a two line spectrum with $\alpha_H = 1.8$ G (Figure 2). The third variety of signal obtained from brain lipid extracts of PBN treated rats was a three line spectrum with $\alpha_N = 15.8$ G, but lacking a detectable proton based hyperfine splitting (Figure 3), similar to the one obtained by other investigators from a gerbil ischemia-reperfusion model under different instrument settings.[11] The species of the free radical adduct of PBN could not be characterized. However, Oliver *et al.* attributed their signal as being possibly due to a one carbon-centered free radical forming spin adduct, oxidized to form a nitrone, which in turn can trap another carbon centered free radical to form a nitroxide lacking a proximal proton.

The initial 10 minute collection (Control) did not produce any EPR signal in all the animal groups, suggesting that ·OH radical is not produced in detectable

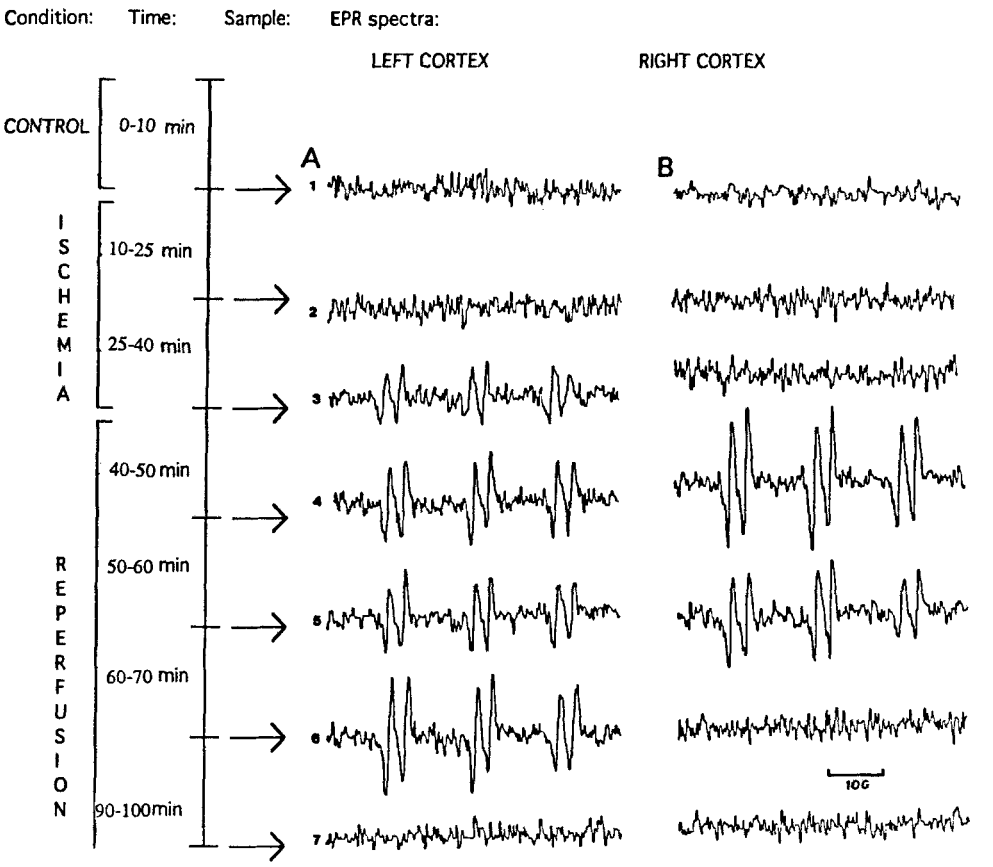

FIGURE 1 EPR spectra obtained from cerebral cortical superfusate samples from a rat at different time points of brain ischemia reperfusion using POBN as a spin trapping agent. Superfusate samples were obtained from the left (A) and right (B) cortical cups respectively. The details of instrument setting and signal characteristics are provided in the text. The left hand side panel indicates the experimental conditions and timings of collection of the superfusate samples.

amounts under normal conditions. It also suggested that there was no pseudoradical adduct formation in the aCSF. In the group of rats subjected to 15 minute ischemia, EPR signals characteristic of ·OH radical were obtained in the 15 minute ischemia period collection (Figure 4). During reperfusion similar signals were obtained but with diminished intensity. The reduction in signal intensity was significant in the 10–20 min reperfusion collection ($p < 0.01$) and returned to baseline values by the 20–30 min reperfusion collection. In the 30 minute ischemia group there was also production of EPR signal characteristic of ·OH radical beginning from the first 15 minute collection. The intensity of the signal increased during the next 15 minute collection and continued to do so in the first reperfusion collection. The EPR signal intensity in the following collections declined and was not detectable after 90 minutes of reperfusion. The results were comparable when DETAPAC was include in the aCSF. For this reason DETAPAC was not used in the superfusate in all

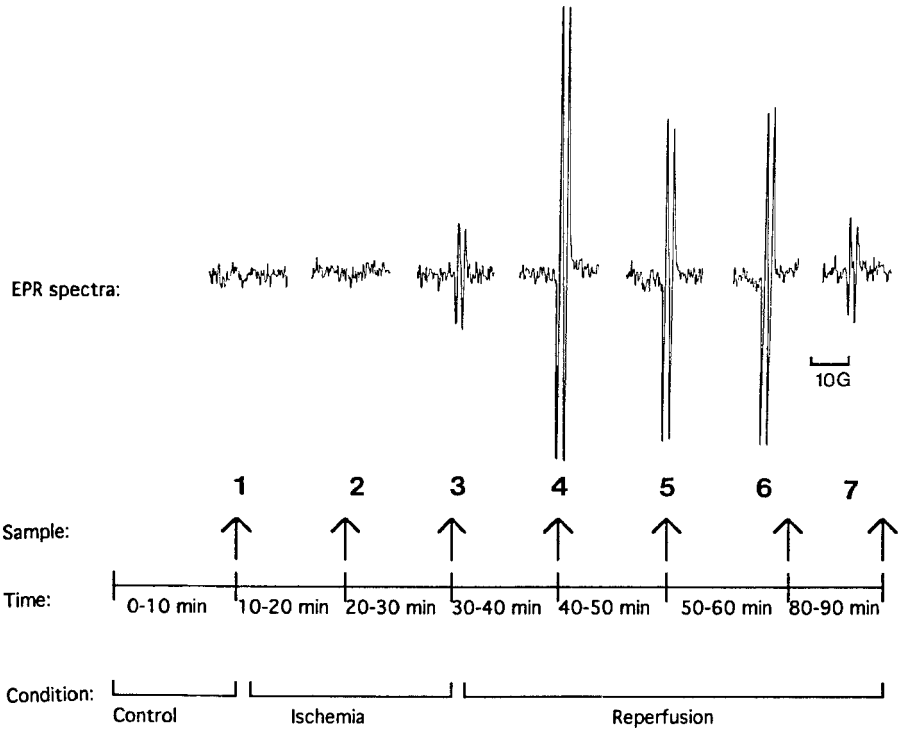

FIGURE 2 EPR spectra characteristic of ascorbyl radical obtained from cortical superfusate samples taken at different time intervals from a PBN treated rat subjected to cerebral ischemia-reperfusion. The bottom panel indicate the experimental conditions and timings of collection of the superfusate samples.

experiments. The EPR signals were always comparable in aCSF from both hemispheres (Figure 5).

Since the animals subjected to 30 minutes of ischemia demonstrated a somewhat sustained release of ·OH radical, a similar protocol was followed for all rats administered with PBN. Administration of 100 mg/kg PBN systemically did not seem to produce any significant change in intensity of the signal attributed to ·OH radical during the ischemia period (Figure 6). However signal intensity declined significantly ($p < 0.001$) beginning in the first reperfusion sample and was only slightly above the threshold level for detection in the remaining period of reperfusion. Two out of six animals revealed the ascorbyl radical signal during reperfusion. In the animals administered PBN systemically and as well as topically (100 mM) in aCSF there was a significant reduction in signal intensity during ischemia ($p < 0.001$) in comparison with both the control and systemically administered PBN experiments. No EPR signals characteristic of ·OH radical were generated by the CSF samples obtained during the entire period of reperfusion. In all the animals in this group, some of the ischemia as well as the initial reperfusion EPR spectra revealed the ascorbyl radical signal. These signals were inconsistent and of variable intensity.

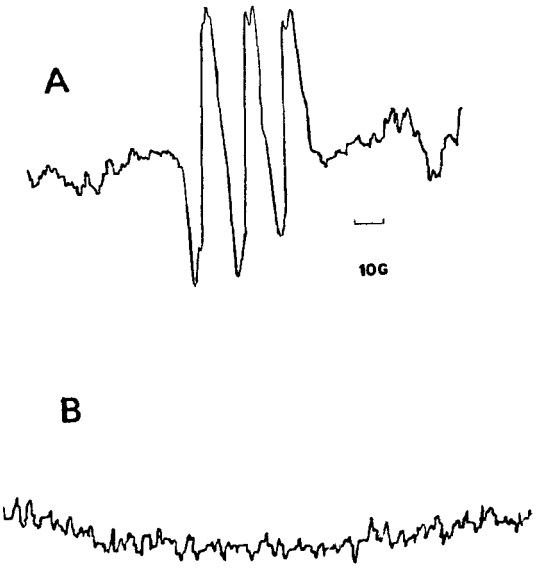

FIGURE 3 EPR spectra obtained from a lipid extract of rat brain subjected to ischemia-reperfusion (A) and a sham operated control rat (B). Rats treated with PBN systemically (100 mg/kg IP). The extraction procedure, the EPR instrument set up and the EPR signal characteristics are detailed in the text.

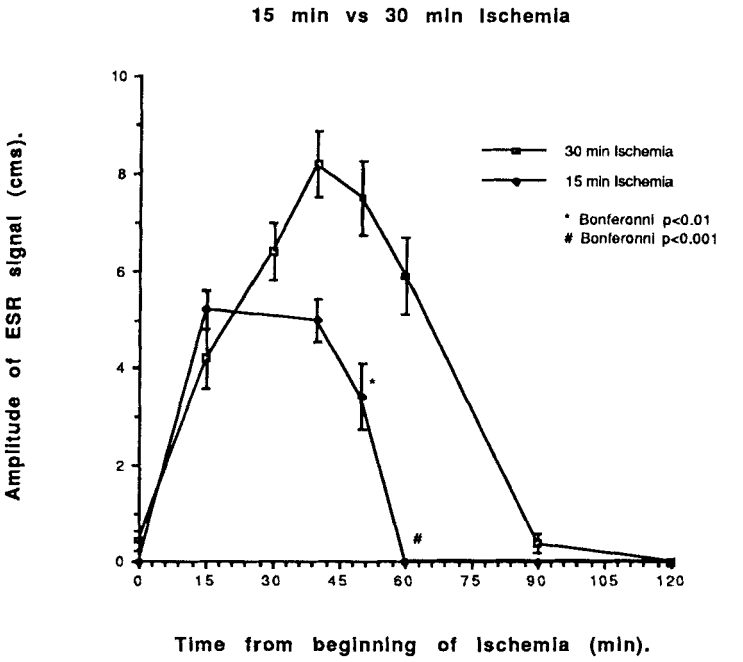

FIGURE 4 Variation of EPR signal intensity with time in rats subjected to 15 min (n = 3) vs. 30 (n = 6) min ischemia followed by 90 min reperfusion. The EPR signal intensity was measured peak to peak and expressed in cm.

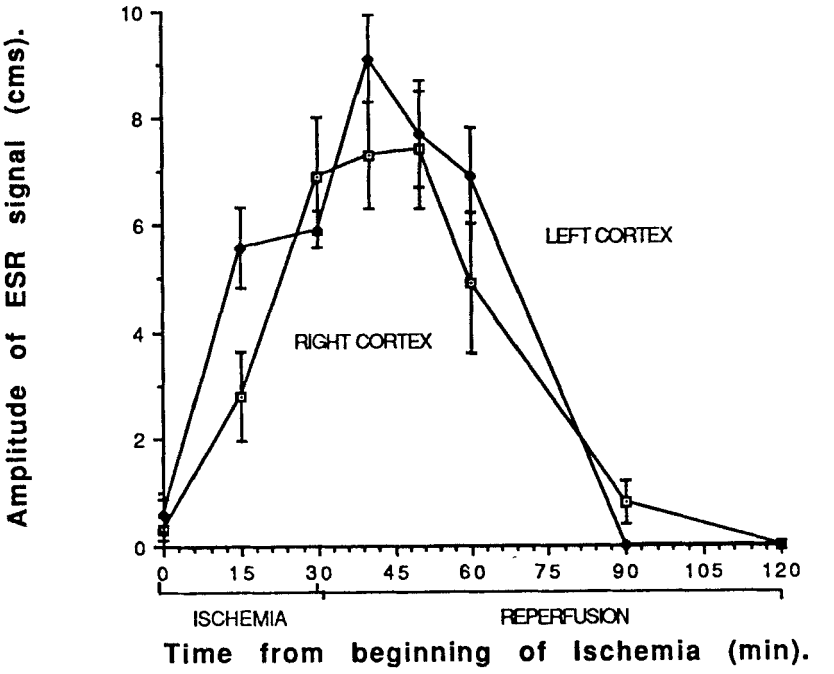

FIGURE 5 Variation of EPR signals with time in cortical superfusate samples obtained from right and left cortical cups. The rats (n = 6) were subjected to 30 min ischemia followed by 90 min reperfusion. The EPR signal intensity was measured peak to peak and expressed in cm. The EPR signals were symmetrical and did not show any significant inter-hemispheric variation at any time interval.

DISCUSSION

EPR in conjunction with spin trapping has been used to detect *in vivo* free radical production in various organ models.[12] In the brain ischemia-reperfusion injury model, the technique most commonly used involves the administration of a spin trap (PBN) systemically, followed by recovery of the spin adduct after homogenization of the brain and its extraction from the homogenate.[11,13,14] Our experience with a similar procedure is that PBN forms a poorly characterized free radical adduct (Figure 3). However, a criticism that can be leveled against such a procedure is that tissue homogenization and organic extraction, on account of the lengthy period of sample manipulation, introduces the possibility of artifacts from *in vitro* lipid peroxidation.[15] Recently the microdialysis technique, using POBN as a spin trap, has been used as an interesting alternative.[9] However the microdialysate samples used to detect spin adducts would entail the removal of spin trap products from their immediate extracellular environment, which might induce their further degradation (see Cheng *et al.*, 1993).[15] Lange *et al.*, in 1990 had also detected *in vivo* free radical production in a pig ischemia-reperfusion model by introducing the spin trap (PBN) through a cannula inserted into the lateral ventricle and then recovering spin adduct from CSF samples drawn from the cisterna magna.[16] Both studies suggested that free radicals were released into the CSF during ischemia-reperfusion.

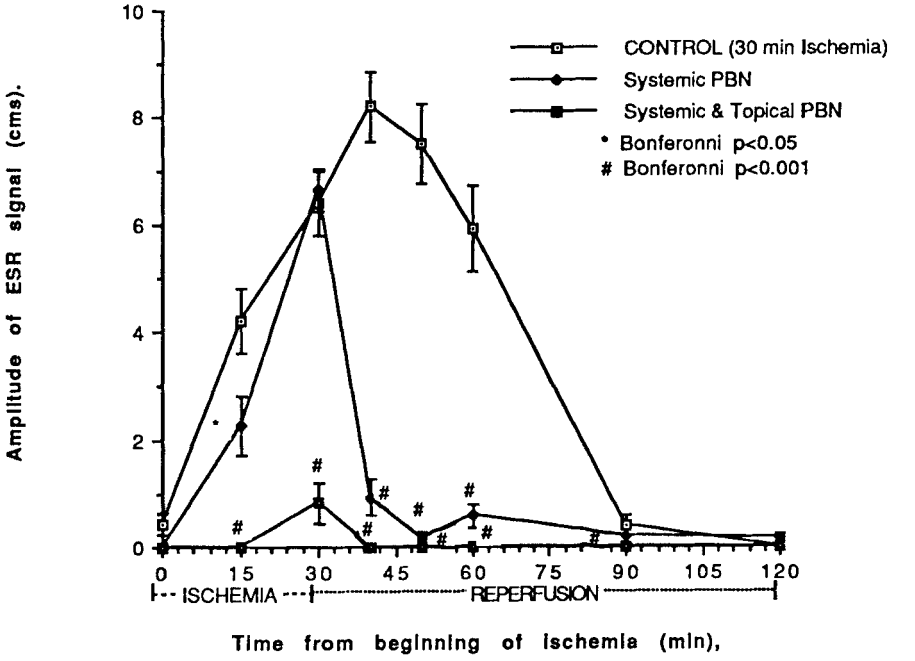

FIGURE 6 Variation of EPR signal intensity with time in rats subjected to 30 min ischemia followed by 90 min reperfusion. The EPR signal intensity was measured peak to peak and expressed in cm. Three groups of animal include (A) Control (n = 6), (B) treated systemically with PBN (100 mg/kg IP) (n = 6), and (C) rats treated with PBN both systemically (100 mg/kg IP) and topically (100 mM via aCSF) (n = 4). A fourth group of animals (n = 8) that were treated the same as the controls except that their aCSF contained 100 μM DETAPAC, gave results similar to the control group and hence are not depicted.

Taking a cue from the Zini *et al.* study, we have used POBN as a spin trap agent and administered it both systemically (IP) and topically in cerebral cortical cups to improve our ability to detect *in vivo* free radical production during ischemia-reperfusion of rat cerebral cortex. On account of its lower lipophilicity, POBN[15] may serve as a more effective spin trap in the aqueous solution of aCSF as compared to PBN. The cortical cup technique proved to be technically appropriate and allowed for several samples to be collected, yielding a temporal profile of free radical production in each cerebral cortex of the rat brain ischemia-reperfusion model.

Our results were similar to those seen in the microdialysate samples by Zini *et al.*[9] However unlike the Zini *et al.* study, POBN did not form pseudoadducts in the absence of chelating agent DETAPAC. Thus whereas Zini *et al.* routinely included DETAPAC in microdialysis perfusate, this was not neccesary in our experiments. Increasing the period of ischemia from 15 min to 30 min resulted in an increased and more sustained ·OH radical release during reperfusion. The pattern was similar to that seen in a gerbil model which utilized hydroxylation of salicylate as an indirect index of ·OH radical production.[8] A study of the temporal profile of EPR signal amplitude, which was used as an index of amount of ·OH radical released in aCSF, revealed that although ·OH was produced during

ischemia it reached a peak in the superfusate sample collected after 10 minutes of reperfusion. This is in agreement with the notion that the major free radical mediated damage is produced during reperfusion and is probably related to reoxygenation of the brain.[17]

PBN, a spin trap agent has been found to possess applications for the detection as well as suppression of free radical production in a brain ischemia-reperfusion injury model, on account of its ability to form free radical adducts. When administered systemically it is known to penetrate the blood brain barrier and readily distribute in the neuronal tissues on account of its lipophilicity.[15] In our study, intraperitoneal administration of PBN, although it apparently did not alter the ·OH radical production during the period of ischemia, greatly attenuated reperfusion induced ·OH radical production. This was in agreement with the view that PBN mediates its neuroprotective action by trapping free radicals.[6] However, in order to do so it must be present in adequate concentrations at the site of free radical production. The cessation of blood flow during the ischemic episode probably temporarily impeded the process of adequate redistribution of PBN into the rat brain. Accordingly, both ischemia and reperfusion induced ·OH radical production were virtually eliminated by administering PBN topically in CSF in addition to its systemic administration. The brain lipid extraction study (Figure 3) supported the view that the PBN mediated suppression of ·OH radical production was related to its ability to form free radical adduct in the rat cerebral cortex.

Interestingly, in our study the PBN treated animals exhibited a post-ischemia ascorbyl radical signal. Such a signal has been regarded as a marker of oxidative stress.[17] It has been postulated that the release of ascorbic acid is mediated by three distinct mechanisms viz., ascorbic acid excitatory amino acid counter exchange, depolarisation mediated and excitatory amino acid receptor mediated ascorbic acid release.[18] In our preliminary experiments conducted prior to the use of POBN, it had been observed that following administration of PBN both topically and IP, ischemia-reperfusion was associated with the appearance of an ascorbyl radical signal in the aCSF (Figure 2) in the absence of a signal characteristic of the ·OH radical adduct. A release of ascorbic acid from the ischemic brain has been previously been reported.[19] These experiments were not pursued when it was observed that ascorbic acid could be oxidized to ascorbyl radical in the aCSF, in the absence of neural tissues. Apparently there were sufficient levels of contaminant metals in the aCSF to catalyze the auto oxidation of ascorbic acid. It is therefore uncertain as to whether the formation of ascorbyl radical detected in the present experiments was merely the outcome of auto oxidation of released ascorbic acid or if ascorbic acid was involved in generation and recycling of radicals from phenolic antioxidants resulting in ascorbyl radical formation.[20] We speculate that a similar recycling of PBN-OH adduct is possible, and the ascorbyl radical produced thereby is detected in the EPR spectra of the CSF samples.

Thus from this study we conclude that ·OH radical is produced during ischemia and in larger amounts during reperfusion of the rat brain. PBN, a known neuroprotective agent, attenuates the ·OH radical production during ischemia and reperfusion. The neuroprotective effect of PBN is possibly related to its ability to form free radical adducts.

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