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MDL 74,180 reduces cerebral infarction and free radical concentrations in rats subjected to ischaemia and reperfusion

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Abstract

The protective effect of MDL 74,180 (2,3-dihydro-2,2,4,6,7-pentamethyl-3-(4-methylpiperazino)-methyl-1-benzofuran-5-ol dihydrochloride) an α-tocopherol analogue free radical scavenger, against cerebral ischaemia and reperfusion in conscious rats has been demonstrated. Tissue damage following middle cerebral artery occlusion (2 h) and reperfusion (8 days) was decreased by MDL 74,180 (0.1 and 1.0 mg/kg per h) infusion beginning 15 min before the onset of reperfusion and continuing for 2 h into the reperfusion period, in a dose-related manner. Nitroxide radical adducts, characterized and quantified by electron spin resonance spectroscopy, were formed on the addition of spin traps to homogenized rat brain tissue previously subjected to global ischaemia and reperfusion. The primary oxidative chain free radicals form diamagnetic intermediates whose slow homolytic decomposition subsequently yields the observed stable spin adducts. Infusion of MDL 74,180 (1–10 mg/kg per h) beginning 15 min before the induction of global cerebral ischaemia (20 min) until the end of reperfusion (5 min), led to a dose-dependent reduction in the final concentration of spin adducts.

Keywords: Cerebral ischemia/reperfusion; Spin trapping; ESR (electron spin resonance); Antioxidant; Free radical scavenger

1. Introduction

Oxygen-derived free radicals have been proposed to be responsible in part for the cerebral damage and necrosis following ischaemia, trauma and haemorrhage (Kontos, 1985; Hall and Braughler, 1989; Siesjö et al., 1989). Direct evidence for the presence of lipid peroxidation in ischaemia derives from reversible models of forebrain ischaemia which show that increases in conjugated diene and peroxide products are minimal during ischaemia (Watson et al., 1984), but become very evident during the reperfusion period (Yoshida et al., 1985; Bromont et al., 1989).

However, because of the transient nature of the initiating and propagating free radicals, and thus difficulties in their detection and measurement in brain tissue, attempts have been made to correlate the concentration of an exogenously supplied free radical scavenger (antioxidant) with the subsequent protection of cerebral tissue from ischaemic injury (Martz et al., 1989; Liu et al., 1989; Xue et al., 1992).

An alternate and more direct method of demonstrating free radical involvement has been utilised by Kinouchi et al. (1991). A decreased infarct size and brain oedema 24 h after focal cerebral ischaemia were observed in transgenic mice overexpressing human copper-zinc superoxide dismutase, which was not dependent on changes in cerebral blood flow but rather correlated with reduced oxidative stress in the brain tissue, as indicated by high levels of endogenous reduced glutathione (Yang et al., 1994).

The technique of electron spin resonance (ESR) spectroscopy allows direct detection and identification of free radicals persisting in low concentrations ($\geq 10^{-7}$ M) typically. The presence of very reactive radicals (at steady concentrations of $\ll 10^{-7}$ M), may be demonstrated only after conversion to stable long-lived radical adduct species by 'spin-trapping'. Investigators have detected a postischaemic change in the ESR signals of rat (Kirsch et al., 1987; Sakamoto et al., 1991) and gerbil (Oliver et al., 1990) brain after pretreatment with the spin trap α-phenyl-*N*-*t*-butyl nitrone (PBN), which suggests the generation of free radicals.

MDL 74,180 (2,3-dihydro-2,2,4,6,7-pentamethyl-3-(4-methylpiperazino)-methyl-1-benzofuran-5-ol dihydrochloride) was selected from a series of α-tocopherol analogues on the basis of its good radical scavenging properties. An

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IC_{50} of 0.45 ± 0.06 μM was determined for inhibition of spontaneous lipid peroxidation in rat brain homogenate and a relative rate constant for reaction with superoxyl radicals of $10.7 \pm 2.9 \times 10^4$ M^{-1} s^{-1} (Grisar et al., 1995). MDL 74,180 has been shown to inhibit ex vivo lipid peroxidation in mouse brain, demonstrating also its adequate brain penetration, and to reduce the effects of head injury in mice (Grisar et al., 1995). In the present study the protective effect of MDL 74,180 against cerebral ischaemia and reperfusion in conscious rats has been demonstrated, which may be related to its free radical scavenging properties as assessed by an ESR spin trap method.

2. Materials and methods

Male Wistar rats (Iffa Credo, Lyon, France) weighing 250–300 g were used. Water and food were provided ad libitum before and after each part of the experiment. Animals were kept under controlled conditions with respect to light, temperature and humidity.

2.1. Middle cerebral artery occlusion / reperfusion

2.1.1. Surgery

Male Wistar rats were anaesthetised with chloral hydrate 350 mg/kg injected intraperitoneally and placed in the supine position. Throughout surgery the body temperature of the rats was kept at 37–38°C by means of a warming lamp and an isothermal blanket. By means of an operating microscope the right common carotid artery was exposed through a midline incision in the neck. The external carotid artery was dissected further and distally ligated. The internal carotid artery was isolated and separated from the adjacent vagus nerve and its branch, the pterygopal artery was ligated. A nylon mono filament (Archimed 3/8) coated with sylastic (Dow Corning) solubilised in carbon tetrachloride or hexane, final diameter of the thread being 0.25–0.4 mm, was introduced from the bifurcation into the internal carotid artery. The common carotid artery was ligated. The coated filament was pushed along the internal carotid artery and further for a distance of 17 mm until it reached the proximal portion of the anterior cerebral artery, blocking the origin of the right middle cerebral artery (MCA) and occluding all sources of blood flow from the internal carotid artery and the anterior and posterior cerebral arteries. The incision was closed, but 1 cm of thread attached to the filament was left protruding after closure of the incision allowing later reperfusion of the middle cerebral artery via the circle of Willis (cerebral artery circle) since the ipsilateral common and external carotid arteries had been ligated.

2.1.2. Protocol

The middle cerebral artery was occluded for a period of 2 h followed by 8 days of reperfusion. MDL 74,180DA was infused in doses of 0.1 and 1 mg/kg per h beginning 15 min before reperfusion and continued for 2 h into the reperfusion period. The rats were killed and the amount of necrotised tissue was measured after 8 days.

2.1.3. Perfusion staining with triphenyl tetrazolium chloride

At the end of each study the rats were reanaesthetised with chloral hydrate (350 mg/kg i.p.) and the trachea cannulated for artificial respiration with a frequency of 60 strokes/min and a tidal volume of 10 ml/kg. The abdominal aorta was separated and cannulated. The thorax was opened to expose the heart and right atria which was cut. Thirty milliliters heparinized saline (50 IU/ml) containing glucose (600 mg/l) at 60°C was rapidly injected (20 ml/min) followed immediately by 30 ml of 3% tetrazolium chloride in saline also at 60°C (20 ml/min). Fifteen minutes later the brains were fixed in situ with a 10% paraformaldehyde solution and the brains removed and stored in the paraformaldehyde solution containing 20% sucrose for at least 3 days. Sections of 1 mm thickness were cut, photographed and the infarcted area measured by means of an image analysis processing system (Imaging Research, Brock University, St. Catherines, Ontario, Canada). The tetrazolium stained viable tissue red; the necrotic tissue remained white.

2.2. Detection of free radical adducts after ischaemia / reperfusion of rat brain

The general approach followed a methodology previously reported by Sakamoto et al. (1991); however, a number of important modifications and tests of efficiency and validity were incorporated (see 2.2.4/5/6).

2.2.1. Surgery

Anaesthesia was induced by an injection of chloral hydrate (i.p.). Both femoral arteries were catheterised, one artery for blood pressure measurement, the signals from the catheter being transmitted to a Gould RS 3400 recorder and a digital acquisition analysis programme (Ponemah, Storr, CT, USA.). The other femoral artery was used for blood withdrawal and reinfusion. A femoral vein was cannulated for infusion of MDL 74,180. The bilateral common carotid arteries were isolated for the purpose of inducing ischaemia when all other procedures had been completed.

2.2.2. Protocol

After 5 min of stabilisation intravenous (i.v.) infusion of one of the compounds commenced 15 min before the cerebral ischaemia induced by occlusion of both carotids accompanied by blood withdrawal into heparinized syringes to lower the mean arterial pressure to 30 mm Hg. After an ischaemic period of 20 min the carotid occlusion

was released and the blood reinfused. The animal was maintained in this reperfused state for a period of 5 min unless otherwise stated.

2.2.3. Infusion regimes

MDL 74,180 (1–10 mg/kg per h) or saline (vehicle control; 2.3 ml per h) infusion began 15 min before the induction of cerebral ischaemia until 5 min after the onset of reperfusion.

2.2.4. Organic solvent extraction of spin adducts

After reperfusion the rats were decapitated and the brain homogenized immediately in 5 ml Krebs-Ringer phosphate buffer (120 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.3 mM $MgSO_4$ and 10 mM Na_2HPO_4), containing α-phenyl-*N*-*t*-butyl nitrone (PBN; 130 mM), and diethylenetriamine-pentaacetic acid (DPTA 1 mM, in Krebs-Ringer phosphate buffer). The most efficient protocol for extraction of lipidic material and the highest final concentration of the stable free radical adducts required 8 ml of 2:1 v/v chloroform-methanol solution containing diethylenetriamine-pentaacetic acid (3 mM). All solutions were deoxygenated by bubbling with nitrogen. After centrifugation at 4000 rpm for 15 min at 20°C the organic solvent extract was evaporated down under nitrogen and stored at a temperature of −80°C until redissolution in 2:1 v/v chloroform-methanol for measurement of spin adduct concentration by ESR spectroscopy.

2.2.5. Tests of extraction procedures

Tests of the reliability and extraction efficiency were performed using the stable radical 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) as a marker at various aqueous buffer organic solvent (2:1 chloroform-methanol) ratios. At 5 ml organic/5 ml buffered brain homogenate the extraction efficiency was 50–60%. The recovered, directly measured, radical signal level could be increased by the use of smaller organic solvent extraction volumes, but in practice problems with the physical separation of phases limited the practicability. A volume of 5 ml aqueous buffer was essential for the adequate homogenization of each brain. Aqueous, homogenized brain, extracts spiked with 2,2,6,6-tetramethylpiperidine-*N*-oxyl gave, after organic solvent extraction, 85 ± 5% of the signal found for similarly treated aqueous buffer only. Thus, this stable nitroxide radical representative of trapped spin adduct nitroxides is not significantly adsorbed on to brain tissue components or degraded, and partitions well into the organic phase.

Tests of evaporation procedure efficiency and reliability were also made using standard solutions (concentrations of 1–100 μM) of 2,2,6,6-tetramethylpiperidine-*N*-oxyl; ESR nitrogen hyperfine splitting [hfs] 16.4 Gauss, 1:1:1 triplet signal. It was shown that evaporation of 4 ml total organic extraction volume, divided into 2 × 2 ml portions for speed of evaporation, was preferable.

2.2.6. Preparation of samples for ESR studies

One dried extract of a sample pair was re-dissolved in fresh 2:1 v/v chloroform-methanol solvent (200 μl total volume) at room temperature; this solution was then used to dissolve the second member of the sample pair. Care was needed to ensure that all radical content was liberated into the solution from the waxy deposits which had formed during the initial evaporation process (whilst avoiding appreciable evaporation of the 200 μl solvent). The re-dissolved sample of radical adduct(s) was placed in a cylindrical glass ESR tube of circa 2.5 mm internal diameter (liquid depth > 40 mm) for the ESR measurements. The same tube was used for all quantitative measurements of relative radical concentrations.

2.2.7. ESR spectrometer and conditions of spectral acquisition

A modified Bruker 300 ESR spectrometer was operated at 9.7758 GHz frequency at a microwave power level of 45 mW; a 100 kHz modulation amplitude of 2.5 Gauss for estimation of radical concentrations (and of 0.3–1.0 Gauss for studies of spectral fine structure to assist in radical identifications) was used and a 60 Gauss sweep about 3480 Gauss over 5 min.

3. Results

3.1. Middle cerebral artery occlusion and reperfusion

Wistar rats, subjected to 2 h of cerebral ischaemia and 8 days of reperfusion and treated with saline beginning 15 min before the onset of reperfusion, had 158.5 ± 40.4 mm^3 of necrotic tissue. This area was significantly reduced in a dose-related fashion by MDL 74,180, to 108.9 ± 48 mm^3 ($P < 0.05$) and 73.1 ± 35 mm^3 ($P < 0.01$) with doses of 0.1 and 1.0 mg/kg per h, respectively (Fig. 1).

3.2. ESR spectral analysis of α-phenyl-N-t-butyl nitrone spin adducts

3.2.1. Normal pattern of the dominant radical

In all cases using α-phenyl-*N*-*t*-butyl nitrone as the spin trap, the dominant (> 80%) detectable radical species exhibited an ESR spectrum consisting of a 1:1:1 intensity triplet (hfs = 14.6 ± 0.2 Gauss) of doublets (hfs = 2.5 ± 0.2 Gauss) and had a *g*-factor of 2.0057 ± 0.0002 (see Fig. 2); the hyperfine splittings are attributable to interaction of the unpaired electron with one nitrogen nucleus, spin $I = 1$, and one proton, spin $I = 1/2$, corresponding to the hydrogen on the α-carbon, respectively. Smaller (< 1 Gauss) hfs were not resolvable and the *C*- or *O*-centered nature of the trapped initial radical was not distinguishable.

3.2.2. Additional spectral lines, and their origins

ESR lines attributable to radicals in much lower concentration than the major species were detectable, but in

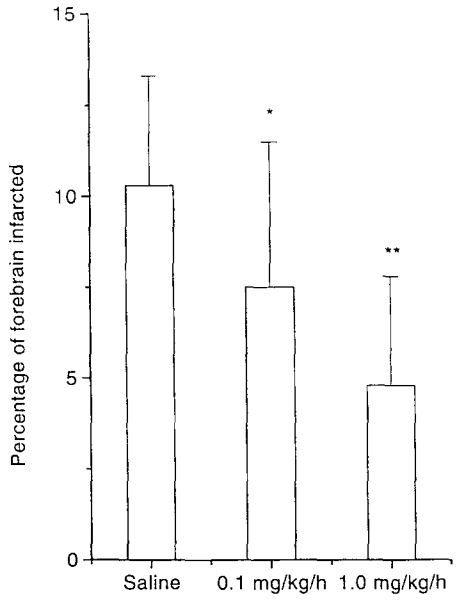


Fig. 1. The effect of MDL 74,180 infusion on the percentage of forebrain infarcted after middle cerebral artery occlusion (2 h) and 8 days of reperfusion. The results are expressed as the means ± S.D. of 8 rats in each group and were compared by means of ANOVA. * $P < 0.05$, ** $P < 0.01$ when compared to saline-treated controls.

general their relative intensities remained in the same proportion to those of the main radical adduct (see Fig. 2). The background signals from the sample tube glass were unimportant. Over a wider magnetic field scan certain lines appearing as additional side peaks on the spin trap adduct spectrum could be seen to be the two high field lines of a four line spectrum having a peak separation of ~ 79.0 Gauss, attributable to a copper or a chromium ($I = 3/2$) protein complex. The intensity of this spectrum showed no correlation with sample type.

3.3. Comparison of different spin traps

Many applications of other spin traps such as 5,5-dimethyl-1-pyrrolidine *N*-oxide, 'DMPO' (e.g. Tsujimoto et al., 1993; Shi et al., 1993; Zang and Misra, 1992) and α-(4-pyridyl-1-oxide)-*N*-*t*-butyl nitrone, 'POBN' (e.g. Chamulitrat et al., 1992; Connor et al., 1994; Sen et al., 1994) in trapping reactive radicals have been reported. At comparable concentrations of the organic spin trap compound (POBN, 65 mg/l; PBN, 65 and 130 mg/l; DMPO, 130 mg/l) added to the whole brain tissue before homogenization, the resulting concentrations in the extracting chloroform-methanol solvent of trapped radicals were similar in all cases. Thus no advantage was to be gained by use of the more water-soluble spin trap α-(4-pyridyl-1-oxide)-*N*-*t*-butyl nitrone in these experiments. Further, in contrast to the usual triplet (hfs 14.6 Gauss) of doublets (hfs 2.5 Gauss) ESR spectrum obtained with α-phenyl-*N*-*t*-butyl nitrone as trap and discussed in (3.2.) above, the use of α-(4-pyridyl-1-oxide)-*N*-*t*-butyl nitrone yielded a simple spectrum (Fig. 2) consisting of a 1 : 1 : 1 intensity ratio triplet (hfs 15.9 Gauss) attributable to appreciable interaction of the lone electron with only one nitrogen nucleus and not with any hydrogen nuclei. This implies that any initial radical adduct has been further oxidized, involving loss of the α-carbon hydrogen atom, and that this product has then acted itself as a spin trap. This difference between α-phenyl-*N*-*t*-butyl nitrone and α-(4-pyridyl-1-oxide)-*N*-*t*-butyl nitrone may relate to the greater solubility of the latter in the aqueous – as compared to the lipid membrane – phase.

The water-soluble spin trap 5,5-dimethyl-1-pyrrolidine *N*-oxide gives complex ESR spectra (Fig. 2), including a 1 : 1 : 1 intensity ratio triplet (hfs 16.1 Gauss) spectrum which is not ascribable to adducts with superoxide anion or hydroxyl radicals, and a spectrum perhaps that of a hydroxymethyl radical adduct (Connor et al., 1994) derived from oxidation of methanol during extraction.

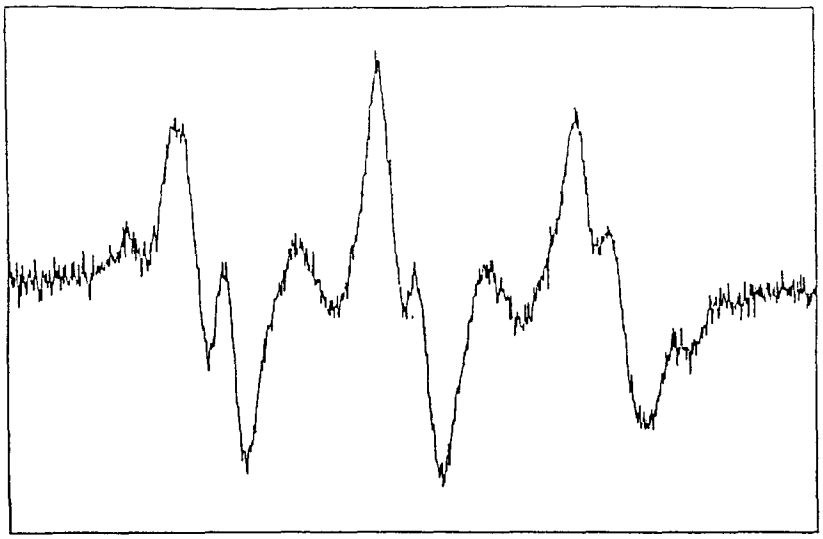
3.4. Elevation of spin-trapped radical concentrations after ischaemia / reperfusion insult (in the absence of anti-oxidants)

Over several series of experiments (including whole/selective hemisphere occlusion of the brain, saline infusion, and infusion/no infusion of spin trap α-phenyl-*N*-*t*-butyl nitrone) the detectable levels of spin-trapped free radicals arising from ischaemic-reperfused brains were enhanced 50–100% above the levels found in the absence of ischaemic insult.

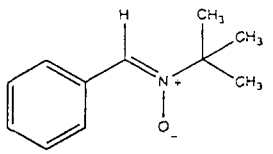
3.4.1. Time course of elevation in radical concentrations after reperfusion

The time evolution of the concentration of free radicals associated with tissue damage arising from ischaemic/reperfusion insult is illustrated in Fig. 3. The duration of the whole brain ischaemia preceding reperfusion was 20 min in each case. α-Phenyl-*N*-*t*-butyl nitrone spin trap was added only in the aqueous buffer used in homogenization of the rat brains after the animals were killed. Since this latter procedure takes a finite time (between 1–2 min) the point nominally taken to be zero with respect to formal reperfusion time involves some short duration (< 2 min) of incidental reperfusion from contact of the brain tissue

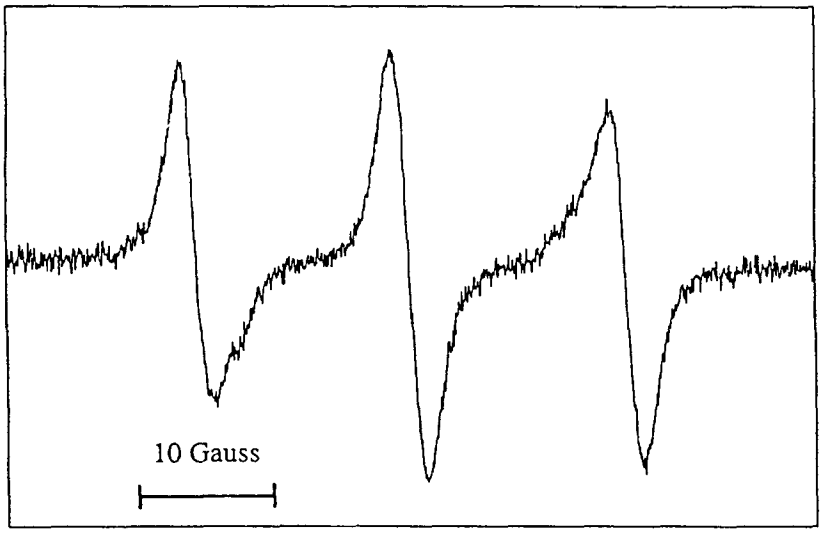
Fig. 2. ESR spectra of spin adducts from rat brain subjected to ischaemia-reperfusion obtained with spin traps. (a) α-Phenyl-*N*-*t*-butyl nitrone, PBN; (b) α-(4-pyridyl-1-oxide)-*N*-tert-butyl nitrone, POBN; (c) 5,5-dimethyl-1-pyrrolidine *N*-oxide, DMPO, added to brain tissue before homogenization. Each spectrum represents a 60 Gauss sweep of magnetic field from 3452 to 3512 Gauss (at a microwave frequency of 9.7758 GHz).



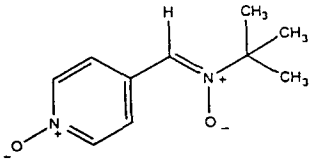
(a)



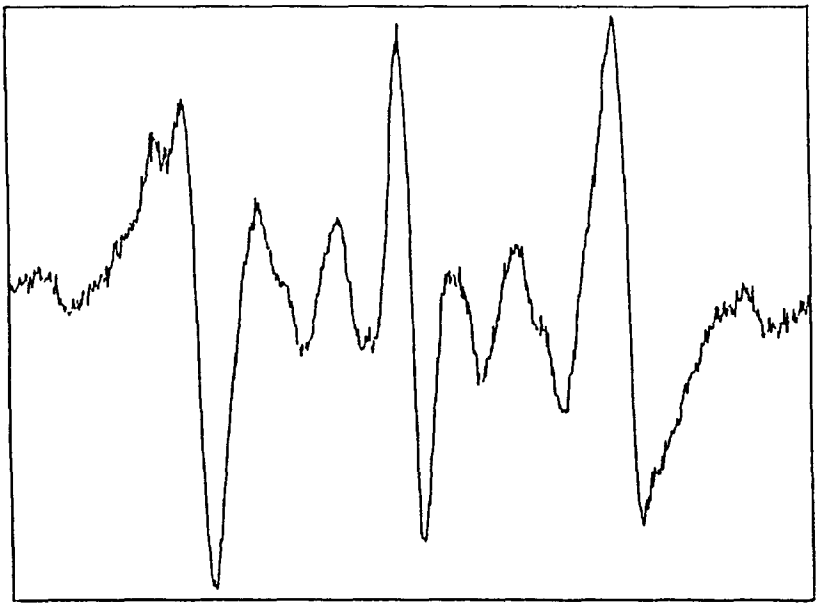
PBN



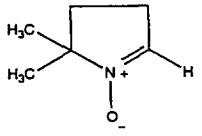
(b)



POBN



(c)



DMPO

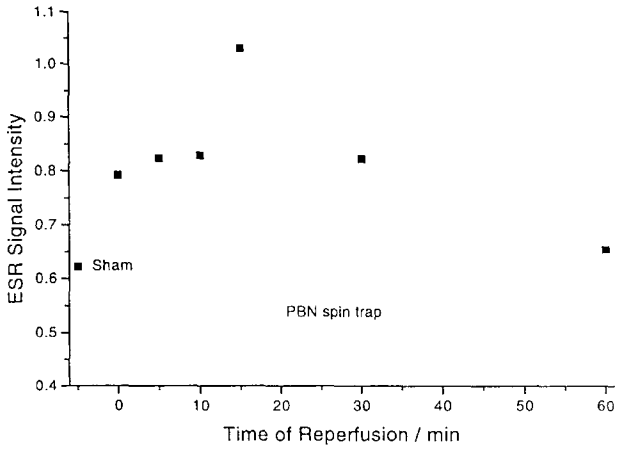


Fig. 3. Time course of the ESR signal intensity of the major spin-trapped adduct of α-phenyl-*N*-*t*-butyl nitrone as a function of reperfusion time following 20 min of global brain ischaemia.

with atmospheric oxygen on excision after the animals were killed.

In these experiments the concentration of trapped free radicals attained a maximum after 15 min of reperfusion following 20 min of whole brain ischaemia, and declined back to the sham level (i.e. no ischaemia, no reperfusion) after 60 min reperfusion.

3.4.2. Scavenging / protective action of antioxidants during ischaemia / reperfusion

In general the levels of trapped free radical adducts (≈ 100 nM), which are linked to the oxidative damage arising from ischaemia/reperfusion insult to the rat brain tissues, were reduced in a dose-dependent fashion by infusion of antioxidant compounds prior to ischaemia (Table 1).

4. Discussion

Results of the present study demonstrate that MDL 74,180 can reduce the cerebral tissue injury resulting from ischaemia and reperfusion and is capable of reducing free radical production under such conditions, which may be responsible for some of the damage. However, the studies reported here provide neither detailed information on the identity of the spin-trapped radicals (since the additions of *C*- and *O*-centered radicals to the nitrone system are distinguishable only by ill- or non-resolved ESR hyperfine splittings arising from hydrogen atoms at least three bonds removed from the oxygen centre of the nitroxide radical adduct), nor their role(s) in membrane lipid and protein oxidation mechanism(s) induced by sudden re-elevation of oxygen levels.

Table 1
Relative concentrations of trapped free radical adducts (in arbitrary units corresponding to the peak-to-peak height of the central doublet in the [first derivative] ESR signals as shown in Fig. 2)

Saline	MDL 74,180	
2.3 ml per h	1 mg/kg per h	3 mg/kg per h
0.404 ± 0.005	0.377 ± 0.005	0.300 ± 0.002

Whole brain ischemia (20 min), reperfusion (5 min); MDL 74,180 or saline were infused for 15 min prior to ischaemia until the end of reperfusion (5 min); α-phenyl-*N*-*t*-butyl nitrone was added in buffer after the animals were killed.

Kinetic considerations indicate that the spin trap method as used in the present (and other) studies does not lead to the *direct* capture of free radicals involved in the primary processes of oxidative degradation. Addition of a spin trap to brain tissue *after* killing of the animal and excision of the brain is unlikely per se to intercept the transient (μs-ms halflife) alkyl, alkoxy, and peroxy organic radicals of the oxidative chain reaction paths. If the spin trap was able to scavenge persistently regenerated, but very low, concentrations of such reactive radicals, the scavenged radical signal should increase steadily with time of contact of the spin trap compound with the brain homogenate after killing. Experimentally such is not the case. A delay of several minutes before addition of spin trap to the extracted brain results in a decrease in ESR signal of the radical adduct.

The evidence suggests that the 'primary radical species' e.g. $RO_2\cdot$, $RO\cdot$ etc., interact with lipid/protein to yield diamagnetic, thermally unstable, intermediates. With time (on a s-min timescale) their thermal homolytic decomposition releases secondary radicals which are spin-trapped directly or react with solvent e.g. methanol, to give other radical species e.g. the hydroxymethyl radical which can be intercepted (cf. Connor et al., 1994). This interpretation explains the insensitivity of the trapped radical yield to the hydrophilic/hydrophobic nature of the spin trap.

Despite the complexity of the oxidative chain reactions, compounded also by the heterogeneous nature of the reaction medium, it is reasonable to assume (1) that the build-up of labile diamagnetic intermediates, capable of subsequent slow generation of free radicals, is a direct function of the level of 'primary radical species', and, therefore, (2) that levels of the radicals ultimately trapped do reflect the magnitude of the original oxidative burst of activity in the brain tissue on reperfusion.

On this assumption the ability of the antioxidants MDL 74,180 to reduce substantially, in an infusion dose-dependent manner, the level of trappable free radicals shows clearly the protective effects of these compounds on general oxidative degradation of brain tissue in vivo subjected to ischaemia/reperfusion insult.

The antioxidants must inhibit the formation of intermediates from the primary reactive radical species, presumably by reaction with the latter to yield more stable antioxidant-derived radicals which decay innocuously i.e. without formation of intermediates capable of later homolytic decomposition.

The ESR-based studies complement and support empirically the assessment of the in vivo protective action of MDL 74,180 in rats subjected to right middle cerebral artery occlusion for 2 h followed by 8 days reperfusion in which the percentage of brain infarcted was reduced by 54% and 31% using doses of 1.0 and 0.1 mg/kg per h, respectively.

There was a 10-fold difference between the doses required to significantly reduce the cerebral infarct size after focal cerebral ischaemia (middle cerebral artery occlusion) and reperfusion and those needed to reduce free radical production following global cerebral ischaemia and reperfusion. There are numerous explanations for this difference, which include the amount of tissue involved in the two insults, and the different end points determined in the two studies.

At present no clinically effective treatment exist for cerebral ischaemia, because the molecular events leading to the modification of vascular reactivity and neuronal damage have only recently been elucidated. The cascade of events probably include energy failure, transmembrane shifts of sodium and potassium, calcium influx, excitotoxicity, oxygen radical formation and lipid peroxidation. With MDL 74,180DA the possibility exists to inhibit free radical propagation chains and although this seems adequate to treat cerebral ischaemia, whether it will prove sufficient in the clinical setting, or rather that a 'cocktail' of inhibitors will be required to block the cascade leading to permanent neuronal damage remains to be seen.

In conclusion MDL 74,180DA is a potential treatment of the tissue damage resulting from cerebral ischaemia.

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