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THE FREE RADICAL TRAPPING AGENT N-*TERT.*-BUTYL-α-PHENYLNITRONE (PBN) ATTENUATES CEREBRAL ISCHAEMIC INJURY IN GERBILS

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Several studies have suggested that oxygen-derived free radicals play an important role in the genesis of ischaemia-induced neuronal damage. We report here that the spin trap agent, N-*tert.*-butyl-α-phenylnitrone (PBN) reduced neuronal damage in gerbils subjected to forebrain ischaemia. PBN (100 mg/kg) administered either 30 min. prior to, or 30 min. after a 5 min. period of bilateral carotid occlusion prevented the increase in locomotor activity observed in saline-injected ischaemic animals and significantly reduced the damage to the hippocampal CA1 pyramidal cell layer observed 5 days post-ischaemia. Telemetry measurements of body temperature revealed that administration of PBN and the induction of cerebral ischaemia were associated with small reductions in body temperature, but these changes were not significant. PBN (100 mg/kg) administered 2 hr post-ischaemia failed to protect against cerebral ischaemia. These findings support the hypothesis of an involvement of free radicals in ischaemia-reperfusion induced cerebral damage and suggest that spin trap agents may be useful for the prevention of cerebral ischaemic damage.

KEY WORDS: Gerbils, cerebral ischaemia, free radicals, spin-trap agents, N-*tert.*-butyl-α-phenylnitrone.

INTRODUCTION

Free radicals have been implicated as causative agents in post-ischaemic injury in a variety of tissues, including the central nervous system.[1-3] Electron spin resonance studies on ischaemic and/or hypoxic rat brain homogenates have demonstrated the possibility that free radical-mediated peroxidation of membranes is initiated during ischaemia and develops during oxygen resupply.[4,5] There is also evidence to suggest that free radical scavengers and antioxidants can protect against ischaemia/reperfusion damage.[4,6-8]

Spin trapping agents are compounds which react with the unstable free radicals to form relatively stable adducts with a typical electron spin resonance (ESR) signal. They are frequently used in ESR studies with the aim of concentrating and revealing the presence of unstable radicals; however there is evidence that they can trap free radicals in biological systems, both in isolated organs[9] and in living animals,[10-12] and can therefore be classified as true free radical scavengers.

N-*tert.*-butyl-α-phenylnitrone (PBN) was selected as the spin trap agent to be used in the present study on account of its established ability to cross the blood brain barrier and capture free radicals generated in the brain by γ radiation or

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ischaemia.[5,13,14] Significant protection against ischaemia-reperfusion cardiac ventricular arrhythmias with PBN and DMPO (5,5-dimethyl-pyrroline-N-oxide), another spin-trap agent, has been reported.[10,15–17] The present studies have been conducted using the gerbil bilateral carotid artery occlusion model, which in addition to histological measurements of CAI hippocampal damage, provides neurological evidence of ischemic injury quantifiable as a measurable hypermotility.[18,19] A preliminary report on the cerebroprotective effects of PBN, administered prior to the onset of cerebral ischaemia, has been published.[20]

METHODS

Male Mongolian gerbils (*Meriones unguiculatus*) (50–60 gm wt.) were obtained from Tumblebrook Farms, West Brookfield, MA. The animals were anesthetized with a mixture of pentobarbital sodium (40 mg/kg) and atropine (0.8 mg/kg) administered intraperitoneally. Under an operating microscope, a midline cervical incision was made and the carotid arteries were exposed and isolated by blunt dissection, with special attention paid to separating and preserving the vagal nerve fibers. A loop of 4–0 braided silk suture was placed around each carotid artery. A sewing needle was used to thread the ends of carotid loops through the neck muscles and the shaved skin of the dorsal neck. One of the exteriorized pair of threads was passed through a small (2 mm) length of polyethylene tube and then tied to its partner. The knots were cemented with cyanoacrylate glue. Another thread (6–0) was attached to each carotid loop and exteriorized through the ventral neck incision. The ventral neck incision was closed with two silk sutures.

Sterile calibrated temperature telemetry capsules (Mini-Mitter Co. Inc., Sunriver, Oregon, Model V) were inserted into the peritoneal cavities of gerbils in all groups, except the saline-treated non-ischaemic controls and PBN pre-ischaemia groups, at the time of the surgery for carotid artery isolation. Records of abdominal cavity temperature were obtained immediately prior to ischaemia (or to drug administration for the non-ischaemic PBN controls) and then at 30, 60 min., 2, 5 and 24 h and 5 days post-ischaemia. After a 48 h recovery period, each gerbil was manually restrained under a heat lamp with fingers on either side of the head and around the abdomen. The carotids were then occluded for 5 min. by pulling on the carotid snares. Schwartz 1″ artery clips were used to maintain the tension on the carotid snares. At the end of 5 min., the artery clips were removed and the snares cut at their point of emergence through the skin and withdrawn from the neck by pulling on the threads emerging from the ventral neck incision. The neck was then gently flexed and extended several times to release the tension on the carotid arteries and re-establish the cerebral circulation. After ischaemia the gerbils were kept at their accustomed environmental temperature of 22°C for 5 days.

The onset of cerebral ischaemia was usually associated with a brief period of panting respiration and body movements followed by quiescence. Successful occlusion of both carotid arteries was evident with the rapid onset of complete bilateral ptosis, and the adoption of a "hunched" posture with the head dropped. Spontaneous motor activity was initially depressed but during the latter portion of the ischaemic period there were intermittent movements of the limbs. Successful reperfusion upon release of the carotid snares was almost immediately evident with the reappearance of head and neck tone and a disappearance of ptosis, and then within a few minutes by a

resumption of motor activity. All animals recovered rapidly and were behaving in a near normal manner within 1–2 h of the ischaemic episode.

Six groups of animals were tested for spontaneous locomotor activity in a Stoelting (Chicago, IL) 31410 Modular Electronic Activity monitor. The activity chambers were housed in a darkened room with masking background noise. Animals were subjected to three sessions of activity measurement. These occurred prior to, and 5 and 24 h after the ischaemic episode (0 h; or after an arbitrary zero time for the non-ischaemic controls). The two groups of non-ischaemic sham operated controls received intraperitoneal injections 0.9% saline ($n = 18$) or PBN (100 mg/kg, Sigma Chemical Co., St. Louis, MO, $n = 12$) dissolved in 0.9% saline. There were 4 groups of ischaemic animals. Data from 3 groups of gerbils which were administered PBN (100 mg/kg) 30 min. prior to ($n = 19$), 30 ($n = 19$) or 120 ($n = 13$) min post-ischaemia was compared with that of saline-treated ischaemic controls ($n = 22$). Only gerbils manifesting symptoms of successful bilateral occlusion were included in the locomotor activity and histopathological studies. Locomotor activity data were collected for 3 × 10 min. periods, following a brief (10 min.) period for acclimation to the activity chamber. Data from the acclimation periods were discarded.

On the 5th day, the animals were anesthetized with pentobarbital sodium and the fore end was perfused via the ascending aorta with 30 ml of 0.9% saline, followed by 50 ml of FAM (40% formaldehyde, glacial acetic acid and methanol, 1 : 1 : 8). The heads were isolated by decapitation and stored in FAM overnight. The brains were then removed and stored in FAM. After sufficient fixation, each brain was sectioned coronally just posterior to, and 3.0 mm posterior to, the bregma. The block of tissue thus formed was embedded in paraffin and a series of coronal sections (10 μ) were cut proceeding caudally through the hippocampus. The sections were mounted on slides and stained with cresyl violet. Slides displaying five representative sections of the CA1 region of the hippocampus[21] were selected under a low power dissecting microscope. The identification labels on each slide were then obscured and new code numbers were assigned by one of us (J.W.P.) to ensure that the histological analysis would be "blind". Five sections from each brain were scored (C.C.H.) by light microscopy for damage to pyramidal cells in the CA1 region of the hippocampus, with ratings being made bilaterally. The histopathological scoring system was based on that used and illustrated in previous studies[18,19] with: 0 = no apparent cell necrosis; 1 = few single cell and/or cell group necrosis; 2 = larger areas of shrunken, necrotic cell groups or missing cells; 3 = most cells necrotic or missing; 4 = virtually complete absence of intact pyramidal cells in the CA1 area. Motor activity data were compared using a one way analysis of variance with Student-Neuman-Keuls or Scheffe tests. The significance of the protection against ischaemic hippocampal CA1 damage with PBN was assessed with the Mann-Whitney U-test. Temperature changes were analyzed with an analysis of variance and Scheffe test. A multiple regression analysis was used to ascertain whether there was any correlation between the temperature changes and histological scores for ischaemic controls and for PBN (0.5 h post-ischaemia) treated gerbils.

RESULTS

(a) *Locomotor Activity*

To evaluate the possible effects of repeated locomotor activity testing (habituation) and of exposure to PBN on locomotor activity, 18 gerbils were treated with saline

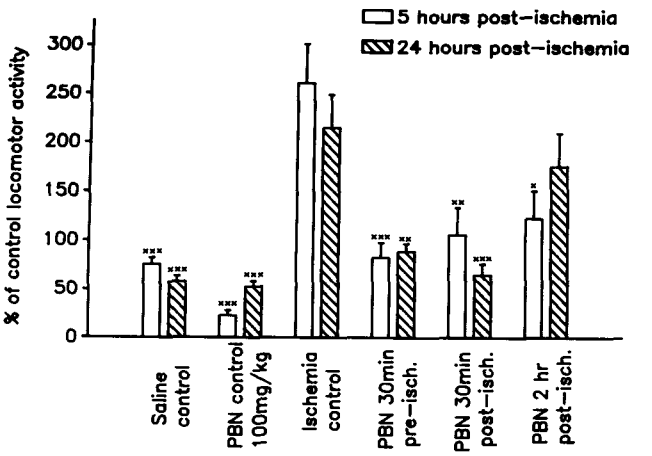

FIGURE 1 Locomotor activities of 6 groups of gerbils. Initial levels of activity for each group are used as the control (100%) for subsequent measures of activity. Each data point represents the mean ± S.E.M. for animals in that group. Locomotor activity of each individual gerbil was monitored for a 30 min. period after a 10 min. interval for adaptation. Control levels of activity for the six groups were: Non-ischaemic saline controls, 148.0 ± 12.0 cpm (n = 18); PBN (100 mg/kg) treated non-ischaemic controls, 131.9 ± 13.9 cpm (n = 12); saline-treated ischaemic controls, 82.2 ± 9.6 cpm (n = 22); PBN treated 30 min. pre-ischaemia, 194.1 ± 21.3 cpm (n = 19); PBN 30 min. post-ischaemia, 56.7 ± 7.1 cpm (n = 19); PBN 2 h post-ischaemia, 70.5 ± 9.2 cpm (n = 13). Significance values for differences from ischaemic controls were calculated by ANOVA with Student-Neuman-Keuls or Scheffe tests.

and 12 animals were injected with PBN (100 mg/kg). Activity in the saline treated gerbils was significantly depressed at 5 h ($p < 0.05$) and 24 h ($p < 0.01$) in relation to the initial basal levels of activity (Figure 1). Declines in locomotor activity from pretreatment basal levels were also observed in the control animals which received PBN.

Following bilateral carotid occlusion, the saline treated group of ischaemic gerbils displayed significantly elevated levels of locomotor activity with respect to both their own level of pre-ischaemic activity as well as to that of the non-ischaemic saline controls. PBM administered either prior to, or 30 min. after, a 5 min. period of cerebral ischaemia significantly reduced the increases in locomotor activity in comparison with those observed in the ischaemic controls at both the 5 h and 24 h testing periods. When administered 2 h post-ischaemia, PBN significantly reduced locomotor activity compared with the increase observed at 5 h in the saline treated ischaemic controls, but the reduction at 24 h was no longer significant (Figure 1).

(b) *Histopathology*

Five days after the ischaemic episode, widespread damage to the CA1 region of the hippocampus was evident in all of the brains of the control ischaemic gerbils. The pyramidal neurons either presented a shrunken appearance with condensed nuclei and minimal cytoplasm, or in many instances had disappeared. Many of these animals displayed a nearly total necrosis or loss of pyramidal cells in the CA1 region. Little neuronal damage was evident outside of the hippocampus. PBN (100 mg/kg) administered either 30 min. prior to, or 30 min. post-ischaemia significantly reduced

TABLE I
Hippocampal CAI "damage" scores

Condition	"Damage" Score
Saline + ischaemia	3.01 ± 0.18
PBN (100 mg/kg) 30 min. pre-I.	1.39 ± 0.28‡
PBN (100 mg/kg) 30 min. post-I.	1.55 ± 0.36†
PBN (100 mg/kg) 2 h post-I.	2.79 ± 0.40

Values are mean ± S.E.M. See Methods section for assessment of damage scores. Significantly reduced in comparison with control ischaemic brains; †$p < 0.01$; ‡$p < 0.001$; calculated by Mann-Whitney U-test.

the degree of neuronal damage and loss in the hippocampal CAI region, whereas when it was administered 2 h post-ischaemia the extent of damage was comparable to that in the saline treated ischaemic controls (Table I).

(c) *Temperature*

In control (no drug) ischaemic gerbils, a small decrease in mean abdominal temperature from pre-ischaemia levels was observed (Table II). The decrease in body temperature was most prominent at 60 min. post-ischaemia (0.9°C; $p > 0.05$). Body temperature recovered to control levels by 2 h post-ischaemia followed by a mild hyperthermia at both 5 and 24 h. PBN administration, in the absence of ischaemia, resulted in a similar, non-significant decrease in body temperature (1.1°C) at 60 min. postdrug. This was followed by a partial recovery to predrug levels at 5 h and by a small hyperthermia at 24 h postdrug.

When PBN was administered to gerbils 30 min. post-ischaemia, body temperature had decreased by 0.3°C at 60 min. post-ischaemia (30 min. postdrug). At 5 h post-ischaemia, body temperature in this group of gerbils had fallen by 1.0°C from pre-ischaemia levels, with recovery at 24 h. In the fourth group of gerbils, abdominal temperature decreased by 1.1°C at 60 min. post-ischaemia (but prior to PBN administration) with some recovery by 2 h. PBN was then administered and body temperature decreased by 2.2°C ($p < 0.05$) at 5 h post-ischaemia (3 h postdrug).

No significant correlations were apparent when multiple regression analyses were used to compare post-ischaemic temperature changes with hippocampal CAI damage scores in either the saline ischaemic controls or PBN (30 min. post-ischaemia)-treated gerbils.

DISCUSSION

It has been widely accepted that neuronal damage in cerebral ischaemia may be in part a result of oxidative damage caused by free radical formation and subsequent lipid peroxidation.[22,23] Free radicals can be generated by several autooxidation reactions and some enzymatic reations. Particular attention has been focussed on the enzyme xanthine oxidase, because of the ability of this enzyme to serve as a source of oxidizing agents such as superoxide radical and hydrogen peroxide. Preliminary evidence for free radical formation in the ischaemic/reperfused brain has been forthcoming from experiments using electron paramagnetic resonance (EPR) techniques.[5,14,24]

TABLE II
Peritoneal cavity temperatures of gerbils measured with implanted temperature telemetry probes

Condition	Time of temperature recording						
	0 min.	30 min.	60 min.	120 min.	5 h	24 h	5 days
PBN 100 mg/kg (no ischaemia) (n = 10)	37.5 ± 0.34	36.7 ± 0.38	36.4 ± 0.36	36.1 ± 0.33	37.0 ± 0.37	38.3 ± 0.32	37.8 ± 0.18
Ischaemia n = 20	37.9 ± 0.18	37.7 ± 0.25	37.0 ± 0.23	37.5 ± 0.32	38.0 ± 0.02	38.2 ± 0.25	37.8 ± 0.39
PBN 100 mg/kg 30 min. post-ischaemia n = 18	37.2 ± 0.27	37.3 ± 0.44	36.9 ± 0.29	36.4 ± 0.29	36.2 ± 0.53	37.1 ± 0.19	37.8 ± 0.27
PBN 100 mg/kg 2 h post-ischaemia n = 7	38.0 ± 0.34	37.9 ± 0.43	36.9 ± 0.46	37.1 ± 0.6	35.8 ± 0.4†	37.0 ± 0.9	37.3 ± 0.49

Values are mean ± S.E.M. 0 min. represents reading taken immediately prior to administration of PBN (no ischaemia) or to the start of a 5 min. period of bilateral carotid occlusion. Significance of reductions in body temperature below initial levels; †$p < 0.05$; by ANOVA with Scheffe test.

A continuous release of free radicals in the brain has been detected during reperfusion periods of up to 60 min following cerebral ischaemia in rats, gerbils and pigs.[6,14,22,25] One potential source of these radicals could be the conversion of hypoxanthine to urate by the O_2^- generating enzyme xanthine oxidase. Xanthine oxidase levels in the rat brain are dramatically increased following cerebral ischaemia,[26] as are the extracellular levels of hypoxanthine and xanthine, which remain elevated for at least 60 min. post-ischaemia.[27] The protective effects of enzymatic quenching of free radicals with superoxide dismutase and catalase[7,8] and of the inhibition of xanthine oxidase[18,28–30] are consistent with the hypothesis that free radicals contribute to cerebral ischaemia/reperfusion injury.

Significant protection against ischaemia/reperfusion injury, assessed as cardiac arrhythmias or myocardial "stunning" has been reported using the spin-trap agents PBN and DMPO.[9,10,16,17] The rationale for using spin trapping agents was that these agents react with free radicals to form relatively stable adducts, thus interrupting the cascade of reactions which ultimately result in membrane lipid peroxidation. PBN was selected as the spin trap agent for the present experiments on account of its established ability to cross the blood brain barrier and capture free radicals generated in the brain by ischaemia or γ-radiation.[5,13,14]

The gerbil model utilized in this study yields behavioral and histopathological data. The advantages conferred by the use of unanesthetized animals include the absence of variability introduced by fluctuating levels of potentially cerebroprotective anesthetics and the absence of anesthesia-induced hypothermia, which can persist for more than 35 min after halothane anesthesia is discontinued,[31,32] as well as the presence of behavioral signs of effective cerebral ischaemia and reperfusion (e.g., complete bilateral ptosis due to lack of blood flow to the orbit with rapid recovery upon reperfusion). Ischaemic damage to the gerbil brain produces large increases in locomotor activity which are correlated with the degree of neuronal degeneration in the CA1 region of the hippocampus.[33,34]. The increased activity may result from a failure of the cognitive functions of the hippocampus with a reduction in the animal's ability to form spatial maps, rather than being attributable to a simple form of motor hyperactivity.[35]

The results of the present experiments show that PBN administered either prior to, or 30 min. after the ischaemic episode, provided a significant degree of cerebroprotection, as manifested by the absence of locomotor hyperactivity. When administered 2 h post-ischaemia, PBN significantly reduced the increase in activity at 5 h in comparison to that manifested by the ischaemic control animals but, at 24 h post-ischaemia the animals were hyperactive. The reductions in locomotor activity observed in the non-ischaemic saline injected control gerbils at 5 and 24 h was, presumably, a result of their familiarization with (habituation to) the activity monitoring chamber.

The conclusion that PBN was cerebroprotective when administered either prior to, or 30 min. post-ischaemia was supported by the histopathological findings. Damage to the CA1 pyramidal cell layer was substantially reduced in these animals. PBN administered 2 h post-ischaemia only slightly reduced the amount of CA1 hippocampal damage, confirming conclusions drawn from the locomotor activity data.

Studies on the metabolism and distribution of PBN in rats indicate that brain levels reach their peak approximately 30 min. after an intraperitoneal injection, have declined appreciably at 60 min., and that its half-life in blood plasma is 2–3 h.[36,37] By

8 h post-administration, plasma PBN levels have fallen to 1/8 of their peak concentration. The locomotor depressant effects of PBN, although apparent 5 h post-administration, should not be evident at 24 h, and indeed the locomotor activity studies demonstrated that there were no differences in the 24 h activity profiles of the saline- and PBN-injected non-ischaemic gerbils.

Ischaemia resulted in non-significant falls in body temperature at 60 and 120 min. of the gerbils in the ischaemia control group and of those which received PBN 2 h post-ischaemia. Falls in gerbil body temperature following cerebral ischaemia, which were enhanced and prolonged by administration of the glutamate receptor antagonist, MK801, have previously been observed.[31,32] For both groups, the hypothermia was most pronounced 60 min. post-ischaemia (0.9° and 1.0° respectively). PBN (100 mg/kg) caused a non-significant fall in abdominal temperature of non-ischaemic gerbils, which reached a maximum 2 h after its administration (Table II). Falls in temperature also occurred in the PBN-treated 30 min. post-ischaemic gerbils, with falls of 0.8 and 1.0°C at 2 and 5 h post-ischaemia. A 2.2°C fall was observed 5 h post-ischaemia when PBN was injected 2 h post-ischaemia. In gerbils which had received PBN 30 min. post-ischaemia, no significant correlation between damage to the CA1 pyramidal cell layer and the degree of post-ischaemic hypothermia was evident.

Busto *et al.*[38] have reported that a reduction in brain temperature to 30°C for 3 h, beginning 5 min. after the resumption of cerebral circulation, conferred a marked protective effect on the rat CA1 hippocampal subfield. If the institution of hypothermia was delayed for 30 min., however, a protective effect was not demonstrable. The mild degree of hypothermia evident 2–5 h post-ischaemia is therefore unlikely to have been a significant factor in the pronounced protective action of PBN administered 30 min. post-ischaemia. The only statistically different data point in our investigation occurred at 5 h post-ischaemia in the PBN 2 h post-ischaemia group (2.2°C, $p < 0.05$) and PBN was not protective when administered 2 h post-ischaemia. Temperature records are not available for the animals which received PBN prior to cerebral ischaemia and a small PBN-induced hypothermia cannot, therefore, be excluded as having played a minor role in the resultant cerebroprotection.[39]

In considering the mechanisms by which PBN exerts its potent cerebroprotective actions against ischaemic damage, hypothermia would, therefore, appear to play a minor role. Although other unknown actions of PBN cannot be excluded, it is assumed that the effect of PBN is primarily related to an interaction with free radical metabolism. Free radical production and release from ischaemic/reperfused brains continue for at least 60 min.[22] An absence of free radicals could account for the lack of effect of PBN administered 2 h post-ischaemia. Free radical formation has been implicated in the release of the excitotoxic amino acids, glutamate and aspartate, from rat hippocampal slices.[40] The excess of glutamate and aspartate released by this mechanism could be one of the factors contributing to the death of neurons after ischaemic injuries. PBN administration, by interrupting free radical formation and metabolism, may result in an attenuation of excitotoxic amino acid release and hence a reduction in ischaemia-induced neuronal loss.

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