Effect of Radical Scavenger *N-Tert*-Butyl-α-Phenylnitrone on Stroke in a Rat Model Using a Telemetric System

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ABSTRACT

We used malignant stroke-prone spontaneously hypertensive rats (M-SHRSP) as a stroke model to explore the effects of the radical scavenger *N-tert*-butyl-α-phenylnitrone (PBN) on stroke. PBN was administrated in drinking water to M-SHRSP. Circadian rhythms in heart rate, blood pressure, and locomotive activity in M-SHRSP were monitored with a telemetric system, in addition to measurement of water intake and body weight. Stroke-onset was assessed by changes in neurological symptoms, water intake, and body weight. Circadian rhythms were basically the same between PBN-treated and control rats several days after stroke onset. Significant differences were seen in blood pressure, relative weight of brain and water intake, heart rate, and locomotive activity between two groups. As a result, no significant difference in age of stroke onset was seen between PBN-treated and control rats, but PBN-treated rats displayed prolonged mean life spans. PBN might be effective in prolonging life span.

INTRODUCTION

The stroke-prone spontaneously hypertensive rat (SHRSP) is a genetic model of hypertension that has a high incidence of spontaneous stroke [1]. Malignant SHRSP (M-SHRSP) is subsequently generated via selective brother-sister breeding between precocious and severely hypertensive SHRSP siblings [2]. These rats develop extremely severe hypertension at an early age, have a higher incidence of cerebrovascular lesions, and possess shorter life spans (approximately 90 days in males as compared with approximately 400 days in normotensive Wister-Kyoto rats, WKY). M-SHRSP serves as a suitable model of hypertension-induced cerebrovascular stroke due to the spontaneous development of stroke at a relatively early age (approximately 80 days) without any need for salt-loading. Stroke-onset in SHRSP has been assessed using neurological signs (behavior), body weight loss, and marked increases in water intake, corroborated by the presence of brain lesions at autopsy [3]. Tabuchi et al. recently showed disturbance of circadian rhythms in heart rate (HR), diastolic blood pressure (DBP), systolic blood pressure (SBP) and locomotive activity using a telemetric system [4]. Variation of locomotive activity, which was counted the amount of action indicating vitality, decreased after stroke-onset. They also demonstrated fluctuation of serum nitrite and nitrate ions (NOx) concentrations at stroke onset in M-SHRSP; acute increase of NO production is associated with neuronal damage [5]. Previously we have measured NO using an *in vivo* microdialysis technique and electron spin resonance (ESR) spectrometry in M-SHRSP, SHRSP and normotensive Wistar-Kyoto rats (WKY) [6]. Brain dialysate NO level was higher in SHRSP than in WKY. NO was not detected in M-SHRSP hippocampus microdialysate after stroke, except after administration of a radical scavenger, N-*tert*-butyl-α-phenylnitrone (PBN). These data demonstrate the usefulness of PBN as an agent to attenuate vascular damage in the M-SHRSP brain [6].

PBN is one of the most useful spin-trapping agents in free radical research [7] and has shown various pharmacological effects in diseases associated with reactive oxygen species [8-17].Stroke onset is thought to relate to vascular damage in the brain caused by oxidative reactions. PBN seems to play an important role in improving the development of stroke, but its effects on circadian rhythm throughout life including pre- and post-stroke have not yet been studied in a rat spontaneous stroke model.

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Employing a telemetric system to investigate further physiological effects of PBN on the development and prevention of stroke seems likely to offer useful data.

In the present study, to determine the effect of PBN on spontaneous stroke in rats, M-SHRSP were dosed with PBN in drinking water and fluctuations in HR, DBP, SBP and locomotive activity were monitored using a telemetric system in addition to measurement of water intake and body weight. This is the first report to explore the pharmacological effects of PBN on circadian rhythm, particularly in a stroke model.

MATERIALS AND METHODS

Chemicals

PBN was purchased from Sigma Chemical (St. Louis, Mo., USA) and other reagents were purchased from Wako Pure Chemical (Osaka, Japan), unless otherwise noted.

Animals and treatments

The experimental protocol received institutional approval. Animal experiments were conducted in accordance with the guidelines for Animal Experiments at the University of Shizuoka. M-SHRSP rats (provided by Dr. Okamoto K, a professor emeritus of Kyoto university, Kyoto, Japan) were maintained by brother-sister breeding under specific pathogen-free conditions with a 12-h light/dark cycle. Rats were housed individually in metabolic cages at 5-weeks-old and allowed ad libitum access to water and standard diet. PBN-treated rats (n=9) and control (n=8) SHRSP rats were dosed with plain and PBN-spiked (0.25 mg/ml) tap water in bottle starting when they were 5 weeks old. Drinking water was changed once a week and measured water consumption. PBN is stable in tap water for at least 1 week [18]. In addition, PBN does not adversely affect the taste of drinking [18]. Stroke-onset was assessed by the appearance of neurological symptoms such as hypokinetic/hyperkinetic syndrome and limb paralysis, and changes in body weight, water intake and circadian rhythm disturbances in HR, blood pressures and locomotive activity [3,4].

Radiotelemetric measurement of HR, SBP, DBP and locomotive activity

Continuous 24-h ambulatory SBP, DBP, HR and locomotive activity were monitored in freely moving rats using the Dataquest IV system (Date Sciences International, St. Paul, MN, USA). Male M-SHRSP rats at 7-weeks-old were anesthetized using intraperitoneal injection of sodium pentbarbital (50 mg/kg; Dainippon Pharmaceuticals, Osaka, Japan). A radiotelemetric probe for measurements of blood pressures, HR and locomotive activity was surgically placed into the peritoneum, a catheter was inserted into the descending aorta [4] and rats were housed individually in regular cages on telemetric receiver pads (1 per rat). The output was relayed from the receiver through a consolidation matrix to a personal computer. Every 5 min, 10 s of SBP, DBP, and HR data were sampled throughout the course of the study, and hourly averages were then calculated until death.

STATISTICAL ANALYSIS

Data are expressed as mean ±standard error of the mean. Analyses were performed using Student's *t*-test (for comparison between pre- and post-stroke) or two-way ANOVA followed by Bonferroni post-test.

RESULTS

Effect of PBN on the age of stroke onset and life span

For the typical rats depicted in Fig 1, the onset of strocke was at the 78 and 80 days age for Control and PBN-treated rats, and life spans were 95 and 115 days, respectively. The mean age at stroke onset was 83.3 ±6.6 days in the Control rats and 88.6 ±10.0 days in the PBN-treated rats (Table 1). No significant difference was identified between groups, showing that PBN did not affect development of stroke. However, mean life spans of Control and PBN-treated rats were 93.3 ±6.7 days and 110.7 ±12.1 days, respectively, indicating that Control rats died ≤10 days after stroke onset, while PBN-treated rats survived significantly longer.

Table 1. Average of stroke-onset, life span and survival span after stroke onset pre- and post-stroke in control and PBN groups.

	Stroke-onset (days after birth)	Life span (days after birth)	Survival span after stroke onset (days)
Control (n=8)	83.3 ± 6.6	93.3 ± 6.7	10.0 ± 2.3
PBN (n=9)	88.6 ± 10.0	110.7 ± 12.1	22.1 ± 4.5
Significance [a]	NS	$p < 0.01$	$p < 0.005$

Data are expressed as the mean ± SE; [a]compared with control and PBN groups; NS, Not significant

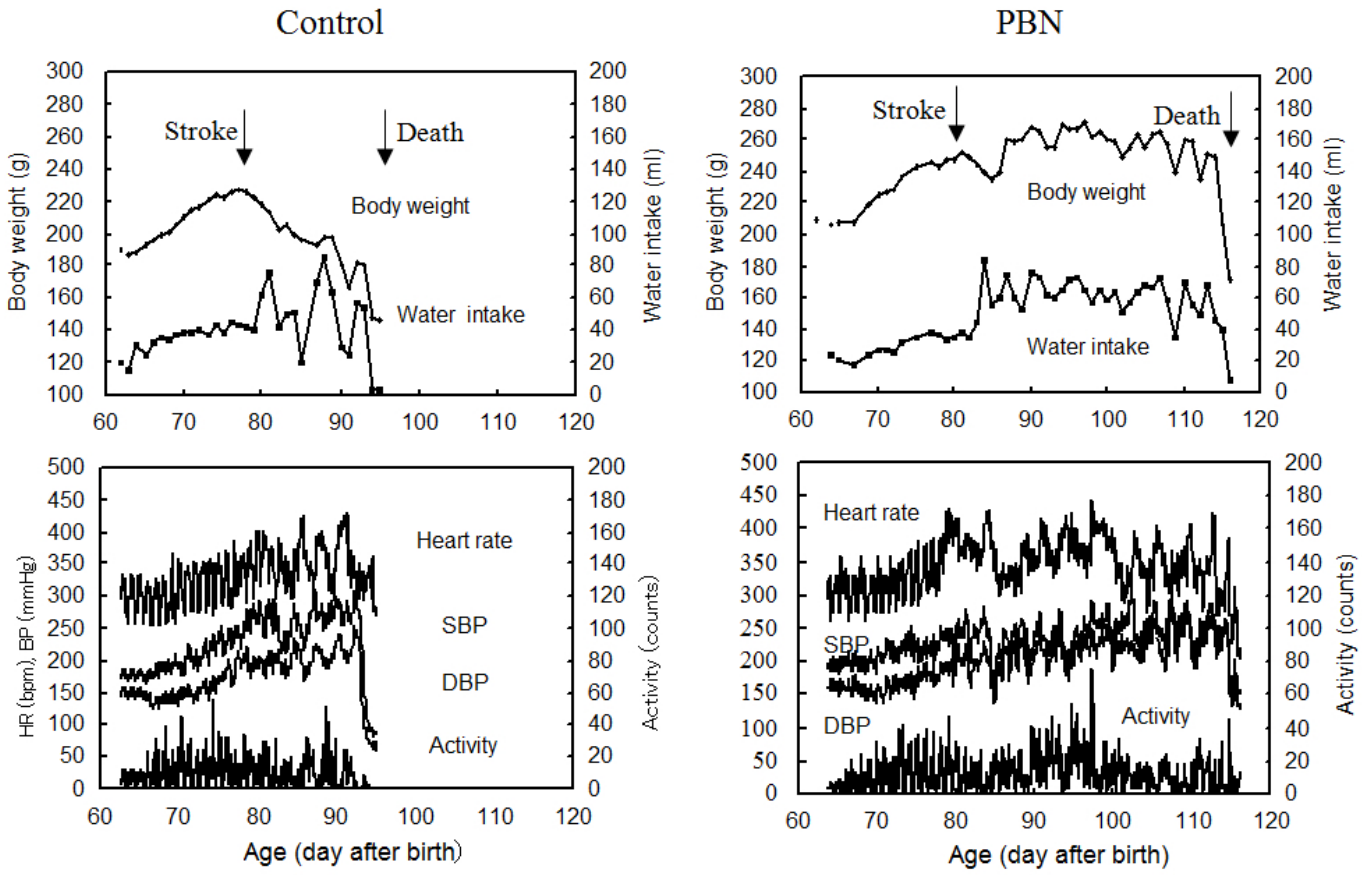

Figure 1. Typical changes in heart rate, systolic and diastolic blood pressure (SBP, DBP), and locomotive activity measured by a radiotelemetry system. Radiotelemetric probes were implanted in the rat abdominal aorta at 7 weeks of age, and then heart rate, systolic (SBP) and diastolic blood pressure (DBP), and locomotive activity were measured every 5 min. Data were averaged for each hour and plotted. Body weight and water intake were measured weekly.

Radiotelemetric monitoring of HR, SBP, DBP and locomotive activity

Typical changes in HR, SBP, DBP and locomotive activity in a representative PBN-treated and control rat until death using a telemetry system are shown in Fig 1. Profiles of circadian rhythms in HR, SBP, DBP and locomotive activity were basically identical for several days after stroke onset in PBN and control groups. Mean HR and locomotive activity at pre- and post-stroke are shown in Table 2.

HR displayed no significant differences at pre-stroke between groups, but a significant difference was seen post-stroke ($p<0.005$). A significant difference between pre- and post-stroke was seen in the Control group but not in the PBN group, indicating that PBN suppressed HR increases even post-stroke. Locomotive activity differed significantly between groups at pre-stroke

($p<0.05$), but not post-stroke. Comparison of pre- and post-stroke displayed no significant differences in either group.

In both groups, SBP and DBP increased with development of stroke, and the changes in SBP and DBP remained extreme for several days after stroke onset. Pulse pressure (difference between SBP and DBP) was smaller in the PBN group than in the Control group. Mean blood pressures (SBP and DBP) were higher at post-stroke than at pre-stroke in both groups (Table 3). SBP both pre- and post-stroke was significantly higher in the PBN group than in the Control group and DBP was significantly higher in the PBN group than in the Control group post-stroke, but not pre-stroke. As a result, pulse pressure at post-stroke was significantly smaller than at pre-stroke in the PBN group, but no significant difference was seen in the Control group (Fig. 1; analysis of Table 3 data).

Changes in body weight and water intake

Typical changes in body weight and water intake are shown in Fig. 1. Water intake in both groups increased with development of stroke and a significant difference was seen between pre- and post-stroke in both groups (Table 4). However, PBN-treated rats consumed significantly less water than controls at pre-stroke, but not at post-stroke. Body weight increased in both groups with age, but after stroke onset, weight of control rats gradually decreased until death (Fig. 1). In contrast, the PBN rat maintained its weight except for a small decrease shortly after stroke. No significant difference in mean body weight was seen between groups, indicating that PBN did not affect growth in rats (Table 4). Brain weight per body was significantly higher in the PBN group than in the Control group, but no significant difference in relative heart weight was seen between groups.

Table 2. Average of heart rate and locomotive activity at pre- and post-stroke in control and PBN groups

	Heart rate (bpm)		Locomotive activity (counts)	
	Pre-stroke	Post-stroke	Pre-stroke	Post-stroke
Control (n=8)	329.1 ± 4.2	352.7 ± 5.9*	11.8 ± 0.63	9.1 ± 1.9
PBN (n=9)	325.8 ± 2.5	337.0 ± 6.7	12.6 ± 0.73	10.84 ± 1.8
Significance [a]	NS	P<0.005	P<0.05	NS

Data are expressed as the mean ± SE. [a]compared control and PBN groups. NS, Not significant. Significance: * p<0.01, for comparison between pre- and post-stroke.

Table 3. Average of systolic blood pressure (SBP) and diastolic blood pressure (DBP) at pre- and post-stroke in control and PBN groups.

	SBP (mmHg)		DBP (mmHg)		Pulse pressure (mmHg)	
	Pre-stroke	Post-stroke	Pre-stroke	Post-stroke	Pre-stroke	Post-stroke
Control (n=8)	202.2 ± 5.1	230.4 ± 12.8*	151.0 ± 6.5	179.8 ± 10.0*	51.6 ± 3.0	50.6 ± 4.7
PBN (n=9)	209.7 ± 4.3	245.1 ± 2.4**	156.4 ± 2.7	197.3 ± 6.0**	53.4 ± 3.8	47.8 ± 5.2*
Significance [a]	p< 0.01	p< 0.05	NS	p< 0.005	p< 0.05	NS

Data are expressed as the mean ± SE. [a] compared with control and PBN groups. NS, Not significant. Significance: * p<0.05, ** p<0.005, for comparison between pre- and post-stroke

Table 4. Average of water intake, body weight and relative weights (g) to body weight at pre- and post-stroke in control and PBN group.

	Water intake (ml)		Body weights (g)		Relative weights (g) to body weight ($x10^{-1}$) [a]		
	Pre-stroke	Post-stroke	Pre-stroke	Post-stroke	at autopsy	Brain	Heart
Control (n=8)	32.9±2.5	40.3±3.4**	218.2 ± 8.5	214.2 ± 12.2	183.5 ± 19.5*	10.9 ± 1.2	5.4 ± 0.32
PBN (n=9)	29.9±1.1	42.7±3.2**	216.6 ± 7.2	218.3 ± 10.9	177.3 ± 17.9*	13.8 ± 1.4	5.4 ± 0.17
Significance [b]	p<0.01	NS	NS	NS	NS	p<0.05	NS

Data are expressed as the mean ± SE; [a] at autopsy; [b] compared with control and PBN groups.

DISCUSSION

We have previously reported the effect of PBN on strokes in a rat model using microdialysis, and found that PBN seems to improve brain function and facilitate the recovery of NO production, although the effects of PBN on stroke onset remained unclear [6]. The present study investigated whether PBN can prevent or delay stroke onset. HR, blood pressure and locomotive activity in M-SHRSP were monitored and water intake and body weight were measured to further explore the actions of PBN. A recent study reported that green tea catechins delayed stroke onset in M-SHRSP, with this effect probably attributable to the inhibition of increased blood pressure, but did not prevent spontaneous stroke in rats [19], In addition, the manner in which catechins inhibit the increase in blood pressure remains to be clarified, and whether catechins prolong life span following spontaneous stroke in rats was not examined. In contrast, the present study showed that PBN prolongs life span without having any preventive effect on stroke. PBN was originally developed as a spin-trapping agent in free radical research and is well known to act as a scavenger for hydroxyl and superoxide radicals that damage blood vessels related to high blood pressure. In genetic model rats, substances other than a radical scavenger may delay the age of stroke onset.

A previous study demonstrated increased water intake with age in normal mice, and PBN-treated mice, which displayed lower drink intakes, could live longer than control mice [18]. In the present study, significant difference was seen in water intake at pre-stroke between the control and PBN groups, which supports the result in Table 4 showing that smaller intake of PBN in pre-stroke prolonged life span compared to control mice. Water consumption is extremely unstable just after stroke and varies widely between individual rats, resulting in the lack of significant difference between groups in post-stroke, though control mice seemed to show large fluctuation in drinking in the stroke period. PBN improved physiological function to decrease water intake and prolong lifespan without significantly affecting growth, as reported previously [18]. Body weight, a marker indicating growth, showed no significant difference between groups throughout life (Table 4), but the ratio of brain weight to body weight was heavier in the PBN group than in the control group, which was supported by the evidence that the brain shrinks and the weight decreases with age. Thus, PBN might facilitate maintenance of brain function. A previous report demonstrated that PBN improves brain function to recover NO production after spontaneous stroke in rats [6]. PBN thus obviously plays an important role in prolonging lifespan related to physiological function through the brain, though PBN prolonged the life span of rats without the effect on stroke. Hypertension is known to cause brain damage, leading to stroke. Although blood pressure was higher post-stroke than pre-stroke in both groups in this study, which is a typical feature in the development of spontaneous stroke in rats, PBN did not show any significant inhibition of high blood pressure (DBP and SBP). Instead, PBN increased blood pressure relative to the control group. The most impressive aspect, as shown in Fig. 1, was that pulse pressure at post-stroke was smaller in PBN-treated rats than in controls. A significant difference was identified between pre- and post-stroke in the PBN rats, but not in the control rats (Table 3). Furthermore, blood pressures (both SBP and DBP) in the control rats seemed much more unstable than in the PBN rats after stroke onset, resulting in earlier death. PBN helped to maintain a stable physiological condition, which might be related to HR. Post-stroke HR was

significantly smaller in the PBN rats than in the control rats, even though no significant difference was seen between pre- and post-stroke in the PBN rats. PBN rats with high blood pressure could prolong life span, in spite of the general mechanism of high blood pressure caused by high HR, suggesting the effect of PBN on the stroke in the rat model may affect baroreflex activity that controls HR through the brain. Blood to other organs is decreased to supply the brain under the low-oxygen condition because the brain demands a great deal of oxygen for life. It is effective for the heart to act at a low level not to consume much oxygen. PBN might increase in systolic blood pressure and control the heart rate to supply the brain with blood, which was controlled by the sympathetic nerve. It has already been proven that PBN penetrates the blood-brain barrier to function in the brain.

In conclusion, the important role of PBN in a spontaneous stroke model in rats might be to stabilize the physiological function and thus sustain life, with the result that PBN prolongs lifespan even after severe stroke. These results provide further information regarding the pharmacological actions of PBN, and should prove useful for medical care.

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