Biosci. Biotechnol. Biochem., **62** (4), 792–794, 1998

Note

A Spin Trap, N-*tert*-Butyl-α-phenylnitrone Extends the Life Span of Mice

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Received August 22, 1997

To characterize the pharmacological effects of N-*tert*-butyl-α-phenylnirone (PBN) on life span, we administered PBN in drinking water to 24.5-month-old mice, and the survivors were counted. Their water consumption and body weights were measured as biological markers. PBN-treated animals as compared with control animals had prolonged mean and maximum life spans. Their water consumption decreased but no significant change was found in their body weights, indicating that the metabolism was improved. Results showed that PBN indeed affects physiological functions and extends life span. We propose that nitric oxide release from PBN may be involved in altering the aging process.

Key words: N-*tert*-butyl-α-phenylnitrone (PBN); oxidative stress: life span; reactive oxygen species; nitric oxide

Oxidative stress caused by reactive oxygen species and free radicals has been implicated as an important factor in the changes of cellular function that occur with aging.[1,2] Thus, oxidative stress increases genetic instability in cells and may represent a factor that destabilizes differentiation.

N-*tert*-butyl-α-phenylnitrone (PBN) is one of the most widely used spin trap agents in free radical research. Numerous reports have demonstrated that PBN protects against, oxidative damage caused by ischemia-reperfusion,[3-5] oxidation of low density lipoproteins,[6] CCl_4-induced rat liver injury,[7] adriamycin-induced cardiotoxicity,[8] and muscle fatigue.[9] In addition, PBN injected into old gerbils resulted in the removal of oxidative damaged proteins to higher levels of multicatalytic protease activities and recovery of the ability to perform in a spatial radial maze to the same levels as young animals.[10] Furthermore PBN has also been found to reverse age-related loss in the stimulation of dopamine release.[11] Recently the effects of PBN on senescence were reported in the senescence accelerated mouse (SAM)[12] and human diploid fibroblast cells.[13] However, these are not a model of the normal aging process. Although PBN is thought to act as an antioxidant, the mechanism is not well understood. We have shown that PBN released nitric oxide (NO) after the reaction with reactive oxygen species *in vitro* and proposed that the release of NO from PBN may play a role in the above-mentioned actions.[14]

In this study, we examined the effects of PBN on the mean and the maximum life spans of normally aged C57BL/male mice.

PBN was purchased from Sigma (St. Louis, Mo, USA). C57BL/6J male mice were obtained from the colony at the Gerontology Research Center (MD, USA). Animals were housed 5 per cage and received Standard Laboratory Rodent Chow (PMI Feeds, Ill. USA) in a stainless steel feeder under standard conditions with a 12 hr light/dark cycle, and were allowed free access to water and the standard diet. The mice at 24.5 months of age were divided into two groups: control and experimental. Each group included 50 mice. Control animals received plain drinking water (tap water) and experimental animals received 0.25 mg/ml of PBN in their drinking water. The drinking water was changed once a week. To discover the effects of PBN, the surviving mice were counted every day. Average weight of mice (initial weight: 40.0±0.5 g) and water consumption of each cage was measured once a week. PBN was extremely stable in tap water and there was no significant change in its concentration for one week (data not shown).

The statistics used were the Peto & Peto long-rank test[15,16] for mean life span and Student's *t*-test for body weight and water consumption.

Recently, Packer *et al.* reported that PBN prolonged the life span of senescence accelerated mice (SAM),[12] which have many features characteristic to mammalian aging[17-19] and are used for studying senescence. In their experiments, daily intraperitoneal injection of PBN (30 mg/kg i.p.) to male or female mice prolonged life span (50% survival rate) from 42 to 56 weeks. Thus PBN was effective for the prolongation of the life span and it is known as a kind of spin trap; this effect was thought to be derived from the scavenging of free radicals formed *in vivo*.

In this study, we examined the effects of PBN, which was orally administrated to normally aged C57BL/6J male mice, on their mean and maximum life span. Figure 1 shows the relationship between the percent survival and life time, where PBN administration was started at 24.5 months. The results of the statistical analysis are shown in Table I. As shown in this table, a significant difference was observed between the two groups, mean life span was prolonged about a month

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Abbreviations: PBN, N-*tert*-butyl-α-phenylnitrone.

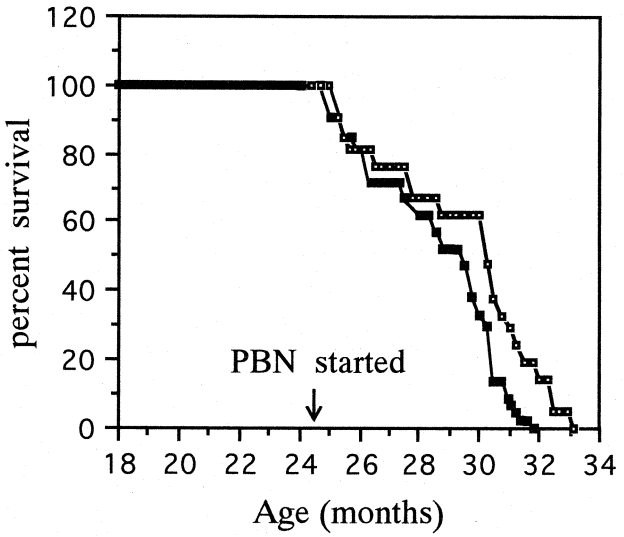

Fig. 1. Effects of Chronic PBN Administration on the Life Span.
□, PBN administered mice; ■, Control mice.

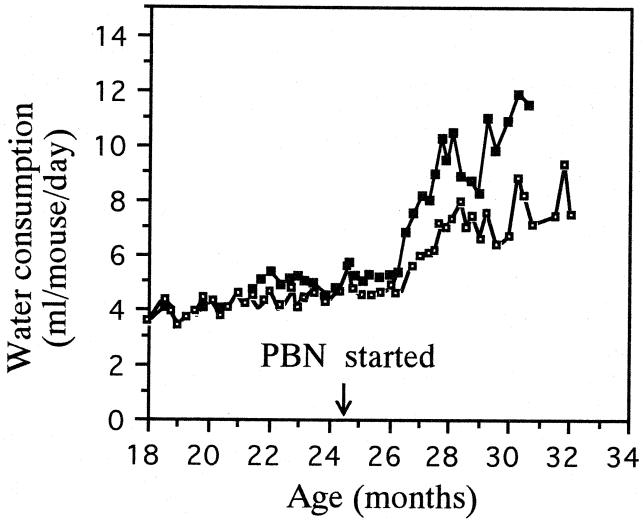

Fig. 2. Effect of Chronic PBN Administration on Water Consumption.
□, PBN administered mice; ■, Control mice.

Table I. Statistical Analysis of the Effects of PBN on Life Span, Weight, and Water Consumption

Biomarker	Control	PBN administration	
mean life span (months)	29.0[a]	30.1[a]	$p<0.005$[c]
maximum life span (months)	31.7[d]	33.3[d]	
weight (g/mouse)	35.0±2.3[b]	34.4±2.9[b]	NS
water consumption (ml/mouse/day)	7.58±2.72[b]	6.09±1.36[b]	$p<0.05$[c]

NS: no significance.
[a] Data are mean (n=50).
[b] Data are mean±SD of survival of mice.
[c] Control vs. PBN administration.
[d] The last survivor.

and similarly, maximum life span was prolonged. In this experiment, PBN administration was started at 24.5 months, so we changed it to 21.5 and 18.5 months to ascertain when the PBN the effect begins. Although their mean and maximum life spans were also prolonged compared with the control, no significant difference was found on them compared with those started at 24.5 months (data not shown). From this result, it was considered that PBN was not effective on young animals because they have enough capacity to protect themselves from oxidative damage, but it become effective on the older ones because they lacked this capacity and as a result, PBN acts as an effective anti-oxidant.

There are several reports about the pharmacological effects of PBN on age-related phenomena.[20,21] For example, an experiment on radial maze patrolling showed that the number of errors made by old gerbils was larger than that by young but it decreased to the same level when PBN was administered to the old ones. However, PBN administration to young gerbils did not have this effect.[21] This is entirely the same tendency observed in our experiment. Another study for the effect of PBN on aging demonstrated that PBN suppressed the senescence of human diploid fibroblast cells caused by oxidative damage.[13] There, PBN delayed senescence and rejuvenated nearly senescent cells. The effect was dose-dependent and was most pronounced for the cells at the stage just before entering senescence. These reports and our result seems to suggest that PBN acted as an antioxidant because it is generally accepted that reactive oxygen species increase with age. However, a number of other antioxidants, such as α-tocopherol, butylated hydroxyltoluene, salicylic acid, and N-acetylcysteine could not extend the life span of the cells.[22]

In our previous papers,[14] we showed that PBN decomposed to generate NO under oxidative stresses such as UV irradiation in the presence of oxygen or oxidation with a Fenton reagent. NO has various pharmacological effects. For example, NO is a kind of endothelial derived relaxation factor and activates guanylate cyclase, resulting in the increase of cGMP. cGMP is a second messenger of signal transduction and activate proteinkinase. As the formation of cGMP in old animals was lower than that in the young (data not shown), it is expected that NO production from PBN in old animals might be helpful for their rejuvenation and prolongation of the life span.

Water consumption of laboratory rodents generally increases with age and it is thought to be caused by age-related impairment in the hydrostatic mechanism.[23] On the other hand, L-arginine-derived NO in the brain acts as an inhibitor of thirst.[24] Therefore, water consumption may be a useful indication of NO production from PBN. Figure 2 shows that water consumption increased with age and a significant difference was observed between two groups with and without PBN administration. PBN administration clearly lowered the water consumption, suggesting that NO was formed *in vivo* from PBN.

Another possibility of PBN effect is dietary restriction, which is also able to extend the life span of rodents, resulting in the loss of body weight.[25] Both

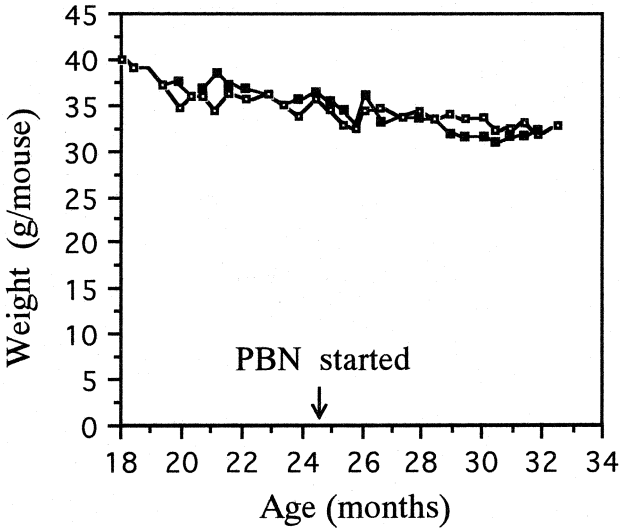

Fig. 3. Effects of Chronic PBN Administration on Body Weight. □, PBN administered mice; ■, Control mice.

curves, of PBN-administered and control groups, showed gradual loss of body weight with age, which is typical pattern in aged animals, but no significant difference was found between them (Fig. 3). This result indicates that the animals of the two groups took almost the same amount of food since PBN had no effect on their appetite. Actually, no difference was observed between their food intake for a week.

From these results and discussion, it was suggested that PBN administration could prolong the life span of mice through the action as an antioxidant and as a NO donor. We are now planning to measure NO generated *in vivo* from PBN by using ESR spin trapping and spectrophotometrical methods.

Acknowledgments

This work was supported by the Glenn Foundation for Medical Research (RGC) and Genox Corp. (KS).

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