Enhancement of Nitroxide-Reducing Activity in Rats after Chronic Administration of Vitamin E, Vitamin C, and Idebenone Examined by an *In Vivo* Electron Spin Resonance Technique

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Rats were given vitamin E (Vit-E), idebenone (ID), or vitamin C (Vit-C) in their food for 2 or 4 weeks. After feeding, the ability of rats to reduce 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol) in terms of the half-life of Tempol was examined as a specific marker. Tempol was repeatedly injected intravenously, and its half-life was serially evaluated by an *in vivo* electron spin resonance (ESR) technique. The radical-reducing ability in rats was enhanced differently by Vit-E, ID, and Vit-C, i.e., slow onset of the ability after Vit-E and ID (lipid-soluble antioxidants) and fast onset after Vit-C (a water-soluble antioxidant).

Key words: ESR; antioxidants; rat; 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol).

INTRODUCTION

The recent development of the electron spin resonance (ESR) system has provided a new tool for examination of the metabolic fate of exogenously administered free radicals *in vivo* (1–7). This system is now applicable to small animals such as rats and mice. In a previous study using an *in vivo* ESR technique, we showed that in rats that were administered iv nitroxyl radical decayed according to first-order kinetics and obtained the half-life of the nitroxyl radical (3). In that study, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol) was used as an exogenous radical. The half-life of Tempol is useful for understanding the nitroxide-reducing activity of living rats.

Vitamin E (Vit-E) (tocopherol) is a major lipid-soluble antioxidant (8, 9), and vitamin C (Vit-C) (ascorbic acid) is a major water-soluble antioxidant (10, 11). Idebenone (ID) is a lipid-soluble quinone-type molecule that seems to improve some clinical aspects of cerebral infarction, cerebral bleeding, and cerebrovascular sclerosis, perhaps by scavenging free radicals (12–14). To our knowledge, however, the radical-reducing activities of these chemicals have only been examined *in vitro* due to the lack of appropriate experimental tools in this regard.

To test the radical-reducing activity of Vit-E, Vit-C, and ID *in vivo*, we administered these agents to living rats in the food. Subsequently, the profiles of the half-life of repeatedly injected Tempol were serially evaluated by an *in vivo* ESR technique.

MATERIALS AND METHODS

ESR Analysis in Animals Given Vit-E, ID, and Vit-C

Thirty-eight male Wistar rats were divided into four groups: control ($n = 9$), Vit-E ($n = 12$), ID ($n = 12$), and Vit-C ($n = 5$). The animals in the control group were fed the standard food *ad libitum*. The animals in the Vit-E group were subdivided into those that were fed the Vit-E-containing food for 2 weeks (Vit-E-2W, $n = 6$) and those that were fed the Vit-E-containing food for 4 weeks (Vit-E-4W, $n = 6$). The animals in the ID group were subdivided into those that were fed the ID-containing food for 2 weeks (ID-2W, $n = 6$) and those that were fed the ID-containing food for 4 weeks (ID-4W, $n = 6$). The animals in the Vit-C group were fed the Vit-C-containing food for 2 weeks. The standard food (CLEA, Tokyo, Japan) contained approximately 5 IU/kg of Vit-E and 25 mg/kg of Vit-C; to this food, Vit-E was added to make the Vit-E-containing food contain 10,000 IU/kg of Vit-E, ID was added to make the ID-containing food contain 1.5 g/kg of ID, or Vit-C was added to make the Vit-C-containing food contain 300 mg/kg of Vit-C.

Upon completion of the feeding schedule, all animals were 13 weeks of age and were subjected to ESR analysis. The details of the *in vivo* ESR analysis have been described elsewhere (3, 15, 16). The ESR system was equipped with a loop-gap resonator (41-mm inner diameter and 10-mm length). The microwave frequency was approximately 700 MHz. Animals were anesthetized with 50 mg/kg of intraperitoneal (ip) pentobarbital, and the animal's head was put into the resonator. The water-soluble nitroxyl radical Tempol (Aldrich, Milwaukee), dissolved in saline at 0.05 *M*, was injected iv via the tail vein at 66.7 mol/kg in 1.33 ml/kg. In a preliminary experiment, a triplet ESR spectrum ($M = +1$, $M = 0$, $M = -1$) of Tempol was observed immediately at the head of the rat after the Tempol injection, and it rapidly decreased to the noise level within approximately 3 min. Therefore, in this study, the injection of Tempol was

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repeated five times at 5-min intervals. After each injection, the peak-to-peak of $M = +1$ was scanned repeatedly until it reached the noise level.

Heart Rate Monitoring

Tempol injection may alter the heart rate, which may influence the half-life of this agent. Therefore, the effects of injection of Tempol on the heart rate were examined. Groups of animals were fed standard ($n = 4$), Vit-E-containing ($n = 4$), ID-containing ($n = 4$), or Vit-C-containing ($n = 4$) foods, each for 2 weeks, until they were 13 weeks of age. Animals were then anesthetized with pentobarbital (50 mg/kg, ip), and Tempol was injected via the tail vein at 66.7 mol/kg in 1.33 ml/kg. The same dose of Tempol was given repeatedly five times at 5-min intervals while monitoring the heart rate. In addition, an equal amount of saline was injected into another group of animals reared on the standard food ($n = 5$).

Blood Levels of Vit-E and Vit-C

Blood levels of chemicals may be closely related to the radical-reducing abilities of animals. Therefore, blood levels of Vit-E and Vit-C were measured. Four animals were given the standard food for 4 weeks. Eight animals were given the Vit-E-containing food for 2 weeks ($n = 4$) or for 4 weeks ($n = 4$). Four animals were fed the Vit-C-containing food for 2 weeks. Upon completion of the feeding schedule, all animals were 13 weeks of age, and their blood was taken for measurement of blood concentrations of Vit-E and Vit-C. Vit-E and Vit-C were measured by high-performance liquid chromatography and the 2,4-dinitrophenylhydrazine method, respectively (17, 18).

RESULTS

After injection of Tempol, an ESR signal of Tempol was observed at the head of living rats. The intensity of the signal ($M = +1$) gradually decreased over the five injections. A semilogarithmic plot of the intensity of the peak-to-peak (Ipp) against time gave a straight line (Fig. 1). The slope gave the half-life for each injection of Tempol.

In the controls, the half-life of the first injection of Tempol was approximately 50 s. A similar half-life was observed in the Vit-E-2W, Vit-E-4W, ID-2W, and ID-4W groups after the first injection of Tempol. However, the Vit-C group showed a half-life of approximately 40 s after the first injection of Tempol, which was significantly smaller than the corresponding value for controls ($P < 0.01$) (Table 1).

After repeated injections of Tempol, animals in all groups showed a gradual increase in the half-life to various degrees. This increase was most prominent in the controls. The half-life of the control group differed from that of the other groups after repeated injection (Table 1). In the Vit-E-2W group, the half-lives obtained after three, four, and five injections were smaller than the corresponding values for the controls ($P < 0.05$ for the third and fourth injections; $P < 0.01$ for the fifth injection),

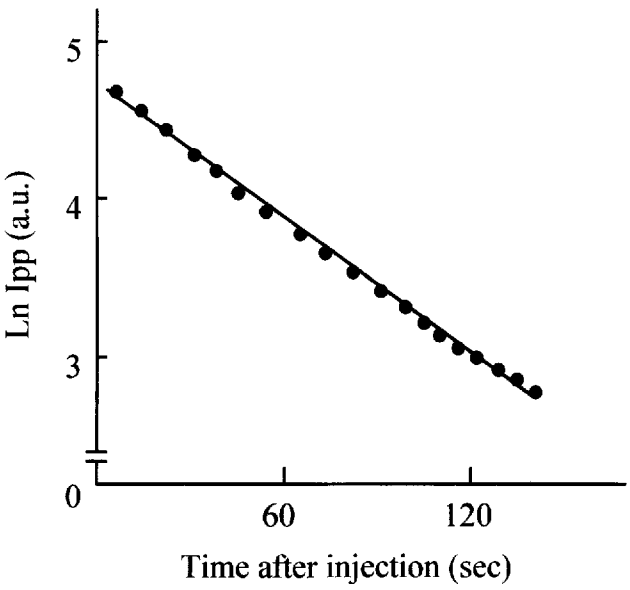

FIG. 1. Decreases in signal intensity (Ipp) against time. The half-life of Tempol was determined from the slope of the straight line.

whereas the Vit-E-4W group showed a significantly smaller half-life than the controls after four and five injections of Tempol ($P < 0.05$ for each injection). The ID-2W group showed a significantly shortened half-life after two, three, four, and five injections of Tempol ($P < 0.05$ for the second and third injections; $P < 0.01$ for fourth and fifth injections), whereas the ID-4W group did so after four and five injections ($P < 0.05$ for each injection). In the Vit-C group, the half-lives after two, three, four, and five injections of Tempol, similar to that after the first injection, were significantly smaller than the corresponding values for the controls ($P < 0.01$ for each injection).

All animals that were fed standard, Vit-E-containing, ID-containing, or Vit-C-containing foods had a heart rate of approximately 400 bpm before the injection of saline or Tempol. Repeated injection of saline alone caused no apparent changes in the heart rate in animals that were given the standard food (Fig. 2). However, all animals that were given standard, Vit-E-containing, ID-containing, or Vit-C-containing foods showed a similar and gradual decrease in the heart rate after injection of Tempol. Eventually, the heart rate decreased to approximately 300 bpm in all animals after five injections of Tempol.

Blood levels of Vit-E and Vit-C in animals that were fed the standard food were 5.1 ± 0.2 μg/ml and 4.3 ± 0.3 μg/ml, respectively (means ± SEM). When the Vit-E-containing food was given for 2 weeks and for 4 weeks, the Vit-E levels in the blood were 7.4 ± 0.4 μg/ml and 7.4 ± 0.6 μg/ml, respectively. Both of these values were significantly higher than the corresponding values for animals given the standard food ($P < 0.05$, unpaired t-test). There was no significant difference in the Vit-E level between animals given the Vit-E-containing food for 2 weeks and 4 weeks. The blood level of Vit-C in animals that were fed the Vit-C-containing food for 2 weeks was 6.8 ± 0.3 μg/ml. This was significantly higher

Table 1
Changes of Half-Life After Repeated Injection of Tempol

Group	*n*	Half-life (s)				
		1st	2nd	3rd	4th	5th
Control	9	50.1 ± 1.3	54.3 ± 1.4	58.9 ± 1.8††	61.5 ± 1.7††	64.1 ± 1.8††
Vit-E-2W	6	49.5 ± 2.3	52.4 ± 3.0	50.6 ± 2.6*	53.0 ± 2.7*	53.8 ± 2.0**
Vit-E-4W	6	50.8 ± 3.3	56.1 ± 4.8	54.6 ± 6.3	54.2 ± 7.0*	57.8 ± 4.8*,†
ID-2W	6	49.3 ± 0.7	49.7 ± 0.5*	51.2 ± 1.2*	52.4 ± 1.1**	51.9 ± 0.8**,†
ID-4W	6	51.9 ± 2.4	55.6 ± 1.3	54.3 ± 2.6	54.1 ± 1.4*	56.8 ± 1.2*
Vit-C	5	39.8 ± 2.1**	43.8 ± 2.5**	48.2 ± 1.7**	46.5 ± 1.7**	48.4 ± 2.7**

Five doses of Tempol were given iv at 5-min intervals. Note the significant shortening of the half-life (* $P < 0.05$; ** $P < 0.01$, unpaired *t*-test) compared to the corresponding value for controls and a significant increase († $P < 0.05$; †† $P < 0.01$) compared to the first value for the same group of animals. Values are means ± SEM.

than the corresponding value for animals given the standard food ($P < 0.01$, unpaired *t*-test).

DISCUSSION

For each injection, semilogarithmic plots of the signal intensity against time gave a straight line. This indicated that the decay of the injected Tempol obeyed first-order kinetics, confirming the results of our previous study (3). Nitroxyl radicals such as Tempol seem to be quickly converted to a hydroxylamine by one-electron reduction in biological systems, losing its paramagnetism (7, 19). The observed rate of loss of Tempol was too fast to reflect excretion in urine. It is most likely that the half-life of Tempol observed here mainly reflects its rate of reduction *in vivo*.

Since Tempol was given iv and since it cannot pass through the blood-brain barrier (15, 20, 21), the ESR signals observed in this study mainly originated from Tempol in the large blood vessels in the head (16). Therefore, determination of the pattern of the heart rate after injection of Tempol seems to be important for understanding changes in the half-life of Tempol. Repeated injection of saline to animals that were fed the standard food had no significant effect on the heart rate. Repeated injection of Tempol, however, resulted in a gradual decrease in the heart rate in all animals that were fed the standard, Vit-E-containing, ID-containing, or Vit-C-containing foods. The pattern of the decrease in the heart rate was similar in all animals, suggesting that the daily consumption of Vit-E, ID, or Vit-C has no apparent effect on the decrease in the heart rate induced by Tempol and that the heart rate is not a decisive factor in the half-life of Tempol.

Our results showed that the blood level of Vit-C is markedly increased after feeding with a Vit-C-containing food, whereas a similar increase in the Vit-E concentration in the blood can also occur with Vit-E-containing food. In accordance with these results, the radical-reducing ability was enhanced in rats given Vit-C or Vit-E, although the profile of the ability of the Vit-C group was different from that of the Vit-E group.

The Vit-C group showed a significantly shorter half-life than the controls after all injections of Tempol, including the first. In contrast, the half-lives in the Vit-E-2W, Vit-E-4W, ID-2W, and ID-4W groups were not different from that in the controls after the first injection. However, animals in the Vit-E-2W and ID-2W groups showed a markedly shortened half-life after two or three injections of Tempol, whereas animals in the Vit-E-4W and ID-4W groups did so after four injections. Vit-E is a lipid-soluble antioxidant and may exert its antioxidant action by reacting with free radicals and by donating labile hydrogen to radicals (9). This reaction seems to occur slowly in the lipid membranes, but not in the aqueous compartment (9). The delayed occurrence of shortening of the half-life of Tempol in the Vit-E groups may be explained by the slow process of the action of Vit-E. It is not certain whether the lipid-soluble nature of ID is involved in the slow decrease of the half-life of Tempol in the ID groups. On the other hand, Vit-C is water-soluble and may function as a potent chain-breaking antioxidant in the aqueous phase (10, 11). In addition, the water-soluble nitroxyl radical Tempol is known to be directly reduced by Vit-C (7). The rapid and protracted shortening of the half-life in the Vit-C group seems to reflect direct interactions of Vit-C with nitroxyl radicals in the vascular system. The findings obtained with Vit-E and Vit-C are in agreement

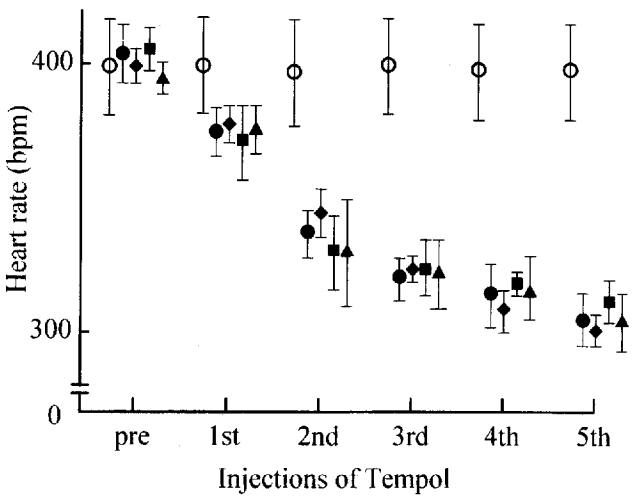

FIG. 2. The changes in heart rate after repeated iv injection of saline or Tempol. Saline injection caused no apparent changes in the heart rate in animals that were given the standard food (○) (n = 5). However, Tempol injections produced a gradual decrease in the heart rate in animals that were given the standard food (●) (n = 4), the Vit-E-containing food (◆) (n = 4), the ID-containing food (■) (n = 4), or the Vit-C-containing food (▲) (n = 4). There were no significant differences in the heart rate after each injection of Tempol among the groups. Values are means ± SEM.

with those of previous *in vitro* studies, that the Vit-E has poorer access to nitroxyl radicals than Vit-C (22).

The period of feeding with Vit-E or ID had no marked effect on the profile of the half-life of Tempol. Instead, the radical-reducing abilities in the Vit-E-4W and ID-4W groups seemed to be weaker than those in the Vit-E-2W and ID-2W groups; the former two groups showed a marked decrease in the half-life after four injections, whereas the latter two did so after two or three injections of Tempol. Since animals that were given the Vit-E-containing food for 2 and 4 weeks showed similar levels of Vit-E-2W in the blood, delayed onset of marked decrease in the Tempol half-life seems to be independent of the blood concentration of lipid-soluble antioxidant drugs. The present findings are compatible with the clinical observation that long-term administration of antioxidant drugs does not always enhance their effects (12, 13).

Additional studies are needed to clarify the actions of antioxidants in biological systems. However, our findings indicate that the radical-reducing ability in rats can be enhanced by chronic administration of antioxidants. The enhancement of the radical-reducing ability induced by lipid-soluble antioxidants (Vit-E and ID) and water-soluble antioxidant (Vit-C) occurred in a different manner: slow onset of the ability after Vit-E and ID and fast onset after Vit-C. These differences were distinguished here by repeated challenge by nitroxyl radicals and the *in vivo* ESR technique.

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