

Potent Radical-Scavenging Activities of Thiamin and Thiamin Diphosphate

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Received 23 May, 2006; Accepted 4 August, 2006

Summary Various radical-scavenging activities of thiamin and thiamin diphosphate (TDP) were found in some *in vitro* experiments. Thiamin and TDP caused considerable suppressive effects on superoxide generation in hypoxanthine and xanthine oxidase system which was measured by a sensitive chemiluminescence method using 2-methyl-6-[p-methylphenyl]-3,7-dihydroimidazo[1,2- α]pyrazin-3-one (MCLA), and their 50% inhibition (IC_{50}) values were estimated to be 158 and 56 μ M, respectively. They also showed the significant suppression against hydroperoxide generation derived from oxidized linoleic acid which was estimated by aluminum chloride method and their IC_{50} values were calculated to be 260 and 46 μ M. They further prevented the oxygen radical generation in opsonized zymosan-stimulated human blood neutrophils which was shown by chemiluminescence method using luminol, and their IC_{50} values were calculated to be 169 and 38 μ M. In contrast, they caused weak but significantly suppressive effects on the hydroxyl radical generation by Fenton reaction which was measured by electric spin resonance (ESR) method, their IC_{50} values were calculated to be 8.45 and 1.46 mM respectively. These results strongly suggest a possibility that thiamin and TDP play as radical scavengers in cell-free and cellular systems.

Key Words: vitamin B₁, thiamin, thiamin diphosphate, radical-scavenging activity

Introduction

Since the discovery of thiamin (vitamin B₁) as antifactor against "Beriberi" disease, various biochemical and pharmacologic activities of thiamin and its derivatives have been reported. For example, a phosphate ester form of thiamin, thiamin diphosphate (TDP) possesses coenzyme activity

for oxidative decarboxylation of alpha-keto acids such as pyruvate, alpha-ketoglutarate and branched chain amino acids [1]. TDP also plays as a coenzyme for transketolase reaction which catalyzes the transfer of glycoaldehyde from keto-sugars to aldo-sugars [2]. These enzyme reactions are associated with cellular bioenergetics involving adenine 5'-triphosphate (ATP) synthesis. As another important pharmacologic activity, thiamin and its derivatives play important

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Abbreviations: CHL, Chemiluminescence; DMPO, 5,5-diethyl-1-pyrroline-N-oxide; ESR, Electric spin resonance; HPX, Hypoxan-

thine; MCLA, 2-methyl-6-[p-methylphenyl]-3,7-dihydroimidazo[1,2- α]pyran-3-one; Opz, Opsonized zymosan; PBS, 10 mM Phosphate-buffered saline (pH 7.4); SOD, Superoxide dismutase; TDP, Thiamin diphosphate; XOD, Xanthine oxidase.

roles in nerve functions. For example, thiamin and thiamin triphosphate are responsible for nerve transmission involving sodium and potassium ion channelling [3]. Furthermore, thiamin improved the metabolic state of the brain of Alzheimer's disease patients, and it was analogous effect compared with the effect on thiamin-deficient brain disease Wernicke-Korsacoff syndrome [4–6]. On the other hand, other researchers found that thiamin and TDP inhibited the glycation of protein using high concentrations of glucose in a diabetic model experiment [7] and other studies indicated that the glycation and other events in diabetic complications are responsible for various oxidative processes such as oxygen radical generation [8, 9]. However, the antioxidant or radical-scavenging activities of thiamin and its derivatives have not been systematically analyzed until present.

On the other hand, we recently found the considerable antioxidant and radical-scavenging activities in the water extract of rice bran and the active principles for these activities were analyzed. As an active protein molecule, we identified the radical-scavenging enzyme such as peroxidase which exhibited the strong antioxidant and antigenotoxic activities [10]. Furthermore, we found another antioxidant and radical-scavenging activities in the low-molecular-weight fraction of the rice bran extract (unpublished data). Since this fraction contained various B group vitamins, we investigated the antioxidant and radical-scavenging activities of B group vitamins by some *in vitro* experiments.

In the present study, we assumed a possibility that thiamin and TDP have potent antioxidant or radical-scavenging activities and detected their preventive effects on lipid peroxidation, hydroxyl radical generation in Fenton reaction, superoxide generation in hypoxanthine and xanthine oxidase system and oxygen radical generation in human blood neutrophils.

Materials and Methods

Chemicals

Thiamin hydrochloride, TDP, luminol sodium salt were purchased from Wako Pure Chemicals Co. (Osaka, Japan). 2-methyl-6-[p-methylphenyl]-3,7-dihydroimidazo[1,2- α]pyrazin-3-one (MCLA) was purchased from Tokyo Kasei Co. (Tokyo, Japan). Hypoxanthine (HPX), xanthine oxidase (XOD), bovine erythrocyte superoxide dismutase (SOD), and yeast cell wall-derived zymosan were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Linoleic acid was purchased from Kanto Chemical Co. (Tokyo, Japan). 5,5-dimethyl-1-pyrroline N-oxide (DMPO) was purchased from Labotec co. (Tokyo, Japan). Other chemicals for experiments were purchased from Sigma-Aldrich Chemicals Co (St. Louis, MO), except for specific chemicals which are described in text.

Assay for superoxide generation in hypoxanthine and xanthine oxidase system

Superoxide generation was measured by a slight modification of the previous method [11]. This method shows about 100-fold more sensitivity than usual cytochrome C method. Initially, a reaction mixture contains 25 μ l of 0.3 M Tris-HCl buffer (pH 7.8) with 1 mM ethylenediaminetetraacetic acid (EDTA), 5 μ l of 20 μ M MCLA, 5 μ l of 3 mM HPX and 1.4 ml of distilled water, phosphate buffered saline (PBS) or vitamin solution, which was preincubated at 25°C for 3 min. The superoxide-dependent chemiluminescence (CHL) generation was initiated by the addition of 5 μ l of XOD solution (60 μ g/ml) to the reaction mixture mentioned above and continued for another 5 min incubation at 25°C. The CHL generations in the presence and absence of SOD (500 U/ml) were compared. The CHL measurement was carried out by a CHL reader (Type BLR-102; Aloka Co., Tokyo, Japan). The experimental result was expressed as the integral CHL intensity for 5 min reaction.

Assay for hydroxyl radical-scavenging activity using ESR method

The hydroxyl radical-scavenging activity was assayed by the modification of the previous Electron Spin Resonance (ESR) method [12]. Distilled water, PBS or vitamin solution (50 μ l), 0.18 M DMPO in distilled water (50 μ l) and 2 mM hydrogen peroxide (50 μ l) were mixed in a glass test tube and finally added with 50 μ l of 2 mM ferrous sulphate in water prepared daily. The reaction mixture was immediately transferred into a quartz ESR flat cell and measured ESR spectra exactly 40 sec after the addition of ferrous sulphate solution using an ESR analyzing apparatus (Type JES-TE200; Nihon Denshi Co., Hamamatsu, Japan). The settings of ESR instrument are as follows: magnetic field; 335.2 mT/power; 8.0 mW/modulation frequency; 9.42 GHz/modulation width; 0.1 mT/sweeping time; 2 min/response time; 0.1 sec/amplitude; 50. The intensity of DMPO-OH spin adduct that is generated from the reaction between DMPO and hydroxyl radical is expressed as a ratio of the signal intensity at the lowest magnetic field to that of manganese ion in water (MnO) used as an internal standard marker.

Assay for the hydroperoxide generation from linoleic acid peroxidation

The amount of hydroperoxide generated from oxidized linoleic acid was measured by the modification of the previous method [13]. One ml of the test solution of thiamin and TDP was mixed with 1 ml of linoleic acid in ethanol and 2 ml of 50 mM phosphate buffer (pH 7.5) in a glass test tube and stood for 4 weeks at 33°C. As a negative control experiment, 1 ml of distilled water or PBS was used in place of test solution. After four weeks reaction, the test solution mixture mentioned above (200 μ l), 250 μ l of 2%

Potassium Iodine (KI) solution in ethanol, 2% aluminum chloride solution and 1 ml of hexane were mixed in a glass test tube and incubated for 5 min at 37°C under dark condition, then 250 μ l of starch solution and 7.5 ml of 10 mM HCl were mixed and shaken vigorously. The solution was centrifuged at 1,500 \times g for 5 min, and the absorbance of the lower layer was measured at 560 nm using Shimadzu UV-265 spectrophotometer.

Assay for oxygen radical generation in human blood neutrophils

Isolation of the neutrophils was carried out as follows. Human venous bloods obtained from healthy non-smoking male donors were recovered in the presence of 3.8% citrate and centrifuged using Mono-poly Resolving Medium (Dai-nihon Pharmaceutical Co., Osaka, Japan) and Krebs-Ringer phosphate solution (KRP, pH 7.4). The neutrophils in the cell layer were recovered and washed two times with KRP by centrifugations and kept in ice until using for the experiment. The viabilities of neutrophils using in reactive oxygen species (ROS) generation experiments were more than 95%. Assay for ROS generation was carried out according to the modification of the previous method [14]. Human neutrophils (1×10^5 cells) were incubated with 50 mM phosphate buffered saline (PBS, pH 7.4) in the presence of luminol sodium salt (250 μ M) at a final volume of 500 μ l. After the preincubation of the cell suspension mixture at 37°C for 3 min, the reaction was started by adding opsonized zymosan (1 μ g/ml) and further incubated for another 30 min at 37°C. Zymosan (5 mg/ml) was opsonized with 50 mM PBS (pH 7.4) including 25% human serum for 30 min at 37°C and washed with 50 mM PBS extensively. Opsonized zymosan (Opz, 1 mg/ml PBS) was kept at -80°C until the use of experiment. For the analysis of effects of thiamin and TDP, they were added to the neutrophils mixture before the exposure of Opz to the cells. The CHL intensity of the cell mixture was recorded continuously during the incubation for 30 min at 37°C using a CHL reader (BLR-201; Aloka Co., Tokyo, Japan).

Expression of experimental results and statistical analysis of data

Experimental result was expressed as the mean and standard error of triplicate assays. Relative inhibitory activity (% inhibition) was expressed as a following equation: (positive control value - vitamin-treated value/positive control value) \times 100%. These values were plotted as a dose-response curve on a semilogarithmic graph in which the concentration required for 50% inhibition (IC_{50}) was determined. The statistical comparison of the data between control and vitamin-treated experiments was carried out using Student's *t* test. A *p* value less than 0.05 was considered to be significantly different.

Results

Suppressive effects of thiamin and TDP on superoxide generation in hypoxanthine and xanthine oxidase system

First, we examined effects of thiamin and TDP on superoxide generation in HPX and XOD system by a sensitive CHL method using MCLA. As shown in Fig. 1, a considerable amount of superoxide was generated in HPX and XOD system as control experiments (distilled water or PBS) which showed the strong CHL intensity. This CHL generation was almost completely scavenged by the exogenous addition of SOD (500 U/ml). When various concentrations of thiamin and TDP were added to the reaction mixture before the start of the superoxide-generating enzyme reaction, the superoxide-dependent CHL generation was suppressed in a dose-dependent fashion from 5 mM to 10 μ M (Fig. 1). In addition, the highest concentration (10 mM) of thiamin or TDP caused almost complete suppression (96.3 or 99.9 % inhibition compared with the control CHL generation). Furthermore, TDP exhibited much stronger activity than thiamin at all the concentrations tested, and IC_{50} values of thiamin and TDP were calculated to be 158 and 56 μ M, respectively. In addition, PBS did not show any significant effects on this experimental system (Fig. 2).

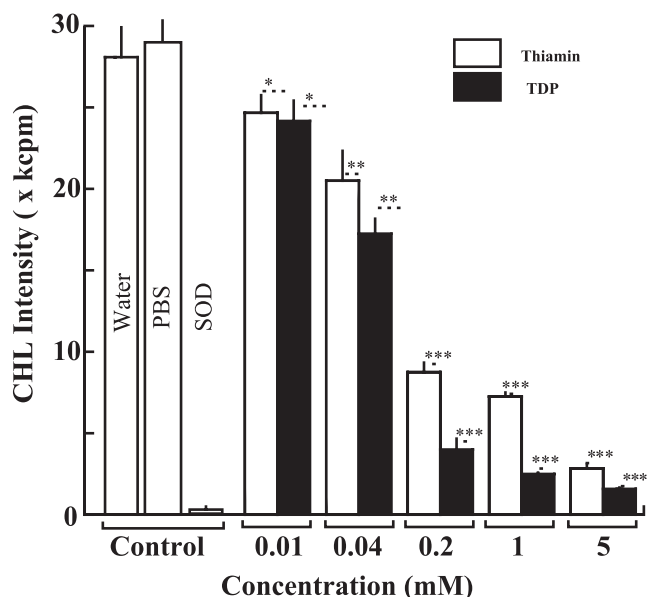


Fig. 1. Effects of thiamin and TDP on superoxide generation in HPX and XOD system. The column and bar show the mean and SD of CHL intensities of triplicate assays. The white and hatched columns represent CHL intensity of thiamin and TDP, respectively. The statistical difference of the results between control and vitamin-treated experiments is analyzed by Student's *t* test. The *p* value is expressed by each asterisk as follows. **p*<0.05, ***p*<0.01, ****p*<0.001.

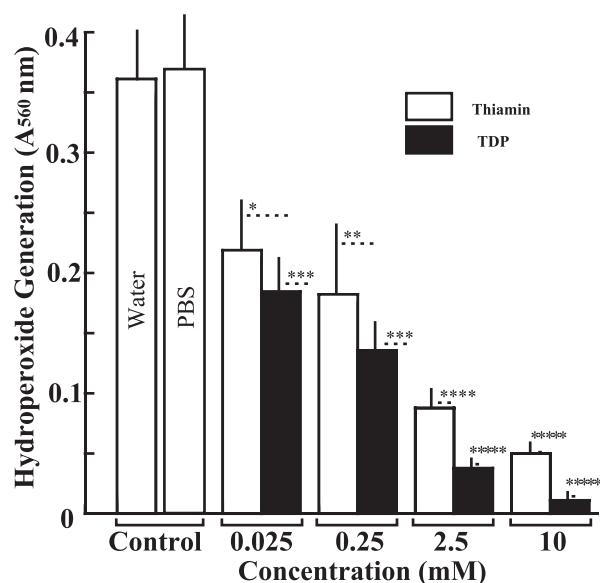


Fig. 2. Effects of thiamin and TDP on hydroperoxide generation in lipid peroxidation. The column and bar show the mean and SD of absorbance by hydroperoxide generation in triplicate assays. The white and hatched columns represent absorbance of thiamin and TDP, respectively. The statistical difference between control and vitamin-treated experiments is analyzed by Student's *t* test. The *p* value is expressed by each asterisk as follows. **p*<0.05, ***p*<0.02, ****p*<0.005, *****p*<0.001, ******p*<0.0001.

Suppressive effects of thiamin and TDP on hydroperoxide generation in lipid peroxidation

We then analyzed the effects of thiamin and TDP on hydroperoxide generation in auto-oxidation of linoleic acid by aluminum chloride method. As shown in Fig. 2, when freshly prepared linoleic acid solution was incubated at 33°C for four weeks, the considerable amount of hydroperoxide was generated by the auto-oxidation of linoleic acid. Thiamin and TDP from 10 mM to 25 μM showed significant suppressive effects on hydroperoxide generation in a dose-dependent manner. TDP exhibited much stronger antioxidant activity than thiamin at all the concentrations tested, and IC₅₀ values of thiamin and TDP were calculated to be 260 and 46 μM, respectively. In addition, PBS did not show any significant effects on this experimental system (Fig. 2).

Hydroxyl radical-scavenging activity of thiamin and TDP in Fenton reaction

Hydroxyl radical generated by Fenton reaction was measured by ESR method using electron signal-trapping reagent DMPO. As a positive control experiment, the strong signals of typical ESR spectra for hydroxyl radical were observed in the presence of ferrous ion and hydrogen peroxide compared with the signal of manganese ion as an internal standard marker and 10 mM thiamin and TDP

showed the strong suppression on the expression of ESR signals (Fig. 3). As a typical data, 10 mM thiamin and TDP showed the strong suppression on the expression of ESR signals (Fig. 3). As shown in Table 1, when pretreated with different concentrations of thiamin or TDP before the generation of hydroxyl radical, a dose-dependent suppressive effect on hydroxyl radical generation in Fenton reaction was observed from 10 mM to 1 mM, which was shown as the relative signal intensity of hydroxyl radical. However, lower concentrations (20 and 100 μM) of these vitamins caused apparently suppressive effects, but not statistically significant effects. TDP showed much stronger suppressive effect than thiamin, and IC₅₀ values of thiamin and TDP were calculated to be 8.45 and 1.46 mM, respectively. This result indicates that hydroxyl radical-scavenging effects of thiamin and TDP are relatively weak compared with their effects in other assays of our present study.

Preventive effects of thiamin and TDP on oxygen radical generation in opsonized zymosan (Opz)-stimulated human blood neutrophils

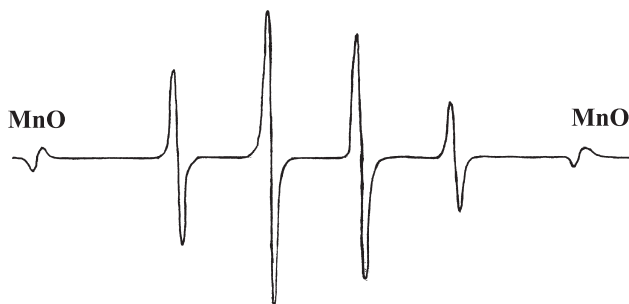
In addition to the cell-free experiments mentioned above, we analyzed the effects of thiamin and TDP on the oxygen radical generation in Opz-stimulated human blood neutrophils as a typical oxygen radical-producing cell system. As shown in Fig. 4, Opz considerably enhanced oxygen radical generation in human blood neutrophils which showed the strong luminol-dependent CHL generation. Another control solution PBS did not cause any significant effects on Opz-stimulated oxygen radical generation. When various concentrations (10 mM–20 μM) of thiamin and TDP were added to the cell mixture system before the exposure of Opz to neutrophils, Opz-induced oxygen radical generation was significantly suppressed in a dose-dependent fashion. Especially, at the highest concentration (10 mM), thiamin and TDP showed remarkable suppressive effects (93.7 and 99.2% inhibition). In this experimental system, TDP showed much stronger activity than thiamin, and IC₅₀ values of thiamin and TDP were calculated to be 169 and 38 μM, respectively.

Discussion

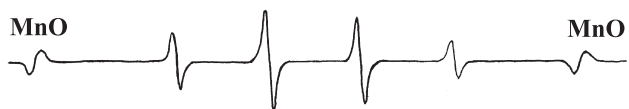
Previously, B group vitamins were considered to be non-antioxidant vitamins. However, recent studies showed that vitamin B6 family such as pyridoxine and pyridoxamine caused suppressive effects on glucose-induced lipid peroxidation and superoxide generation in diabetic model experiments [15, 16]. These results indicate a possibility that some of B group vitamin possess potent antioxidant or radical-scavenging activity.

On the other hand, other investigators reported that thiamin and TDP caused the significant inhibitory effects

(a) Control



(b) 10 mM Thiamin



(c) 10 mM TDP



Fig. 3. Typical ESR data of effects of thiamin and TDP on Fenton reaction. (a) positive control experiment of Fenton reaction using ferrous ion and hydrogen peroxide as explained in Materials and Methods. (b) Effect of 10 mM thiamin on Fenton reaction. (c) Effect of 10 mM TDP on Fenton reaction. MnO shows the position of manganese oxide as an internal standard marker.

Table 1. Suppressive effects of thiamin and TDP on hydroxyl radical generation in Fenton reaction

| | | Relative signal intensity | % Inhibition |
|---------|-------------------|---------------------------|--------------|
| Control | (distilled water) | 13.92 ± 1.68 | – |
| Control | (PBS) | 13.56 ± 1.05 | – |
| Thiamin | 10 mM | 6.12 ± 0.45 (P<0.01) | 56.0 |
| | 5 mM | 8.85 ± 0.69 (P<0.01) | 36.4 |
| | 1 mM | 9.91 ± 1.70 (P<0.05) | 28.8 |
| | 100 μM | 12.04 ± 1.27 (P<0.20) | 13.5 |
| | 20 μM | 12.32 ± 1.08 (P<0.30) | 11.5 |
| TDP | 10 mM | 0.89 ± 0.12 (P<0.001) | 93.6 |
| | 5 mM | 1.89 ± 0.18 (P<0.001) | 86.4 |
| | 2.5 mM | 2.88 ± 0.42 (P<0.001) | 79.3 |
| | 1 mM | 8.63 ± 0.67 (P<0.01) | 38.0 |
| | 100 μM | 10.98 ± 1.53 (P<0.10) | 21.1 |
| | 20 μM | 12.15 ± 1.92 (P<0.30) | 12.7 |

The signal intensity of hydroxyl radical generation is expressed as a ratio of the signal intensity at the lowest magnetic field to that of manganese ion in MnO used as an internal standard. Results are shown as the mean and SD of triplicate assays. The statistical difference of the results between control and vitamin-treated experiments is estimated by Student's *t* test.

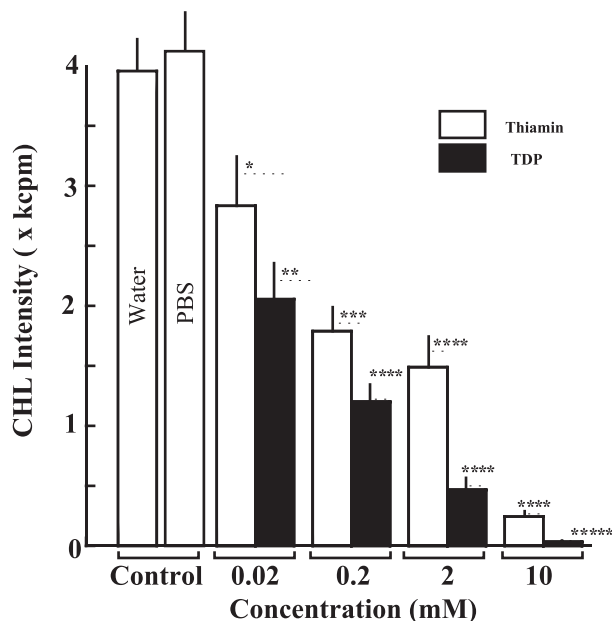


Fig. 4. Effects of thiamin and TDP on oxygen radical generation in Opz-stimulated human blood neutrophils. The column and bar show the mean and SD of CHL intensities generated by oxygen radicals in triplicate assays. The statistical difference of the results between control and vitamin-treated experiments is analyzed by Student's *t* test. The *p* value is expressed by each asterisk as follows. **p*<0.05, ***p*<0.01, ****p*<0.005, *****p*<0.001, ******p*<0.0001.

on protein glycation in a diabetic model experiment [7]. Although the inhibitory mechanism of thiamin and TDP was not analyzed in their report, the other studies indicate that the glycation process of proteins in diabetic complications involves not only Schiff-base formation and Amadori reaction but also another oxidation process such as oxygen radical generation [8, 9]. These observations suggest a possibility that thiamin and TDP have potent antioxidant or radical-scavenging activities. Possibly, thiazole structure in thiamin and TDP may have potent inhibitory activity against the protein glycation because a thiazole derivative (ALT-711) showed the preventive effects on glucose-derived protein cross-linking [17] and improved large artery properties in diabetic rats [18]. Furthermore, another thiazole derivative showed radical-scavenging activity in ethanol-induced oxidative stress in rat alveolar cells [19]. These findings may be associated with the potent radical-scavenging activities of thiamin and TDP. As shown in our present study, thiamin and TDP exhibited the radical-scavenging activities in some *in vitro* experiments.

Previously, other researchers reported a controversial result which showed antioxidant and prooxidant activities of thiamin in some *in vitro* experiments [20]. In their report,

thiamin did not cause significant effects at 0.5–2 mM, but exhibited 32% inhibition at 5 mM against superoxide generation by pyrogallol auto-oxidation. In contrast, our experimental result indicated that thiamin and TDP showed much stronger activities, and their IC_{50} values were 168 and 56 μ M against superoxide generation in HPX and XOD system. Furthermore, in their report, thiamin reversely enhanced the prooxidant activity against microsomal lipid peroxidation at 2.5–10 mM. However, we showed the inhibitory effects of thiamin and TDP on the hydroperoxide generation in lipid peroxidation at 25 μ M–10 mM (Fig. 2). Although the reason of the controversy between our present and their previous results is not clear at present, these different results may reflect the difference of experimental conditions or experimental systems. Possibly, more detailed comparative analysis will be required to estimate exactly these controversial results.

As another interesting finding in our study, the effective concentrations of thiamin and TDP in superoxide generation in HPX and XOD system were relatively low compared with those in the generation of hydroxyl radical in Fenton reaction. As calculated from the experimental result, IC_{50} values of thiamin and TDP for superoxide generation were 158 and 56 μ M, but IC_{50} values for hydroxyl radical were 8.45 and 1.46 mM. These observations seem to be consistent with their effects on oxygen radical generation in Opz-stimulated human neutrophils, in which their IC_{50} values were 169 and 38 μ M, respectively. Opz-dependent CHL generation in human neutrophils using luminol reflects primarily superoxide-generating activity of NADPH-dependent oxidase in the cell membrane [21]. Possibly, thiamin and TDP caused more specific inhibitory effects on superoxide generation than on hydroxyl radical generation. Furthermore, the present finding is consistent with the following observation in the previous biochemical study [22]. A TDP-dependent enzyme transketolase catalyzes the transfer of glycoaldehyde from keto-sugars to aldo-sugars to produce glycolate. This enzyme reaction absolutely requires TDP that is localized at the active site of the enzyme. Interestingly, it was shown that this enzyme reaction was preferably proceeded by superoxide, but not by hydroxyl radical according to the experiments using different radical scavengers [22]. Although the direct interaction between TDP and superoxide was not analyzed in this report, our present finding suggests that TDP localizes in the active site of transketolase and possesses much higher affinity for superoxide than hydroxyl radical.

In addition, thiamin and TDP also showed suppressive effects on hydroperoxide generation in the auto-oxidation of linoleic acid (Fig. 2). Generally, lipid peroxidation consists of different steps such as initiation, propagation and termination steps. Especially, the propagation step contains hydroperoxide generation which is associated with peroxy radical generation

in the initiation step and deprivation of hydrogen atom from linoleic acid [23]. The suppressive effect of thiamin and TDP on hydroperoxide generation from oxidized linoleic acid may be responsible for their inhibitory effects on these reactions in the auto-oxidation of linoleic acid.

Interestingly, our present study indicated that TDP showed much stronger radical-scavenging activity than that of thiamin in all assays (Fig. 1, 2, and 3, Table 1). Although the role of diphosphate in radical-scavenging activity of TDP is not elucidated completely, the metal-chelating property of diphosphate may strengthen the radical-scavenging activity of this compound. For example, since hydroxyl radical generation in Fenton reaction absolutely requires metal ions such as ferrous or copper ion, the metal-chelating property of diphosphate in TDP may contribute to the effective radical-scavenging of TDP. Furthermore, in some cases, lipid peroxidation involves the metal-dependent reaction associated with various oxygen radical generation [24, 25]. The metal-chelating activity of TDP may cause the inhibitory effects on the generation of peroxy radical and hydroperoxide radical.

Somehow, although the more detailed study for radical-scavenging activities of thiamin and TDP should be required, the findings in our present study may give a new biochemical or pharmacologic aspect of these vitamins.

Acknowledgements

The authors would like to thank Dr. Imada, I. (Department of Biochemistry and Molecular Pathology, Osaka City University Medical School) and Dr. Haraguchi H. (Department of Life Science Engineering, Fukuyama University) for useful discussion during the present study.

References

- [1] Cathcart, A.E. and Thurnham, D.I.: Thiamin-Physiology, in *Encyclopedia of Human Nutrition*, eds. By Sadler, M.J., Strain, J.J., and Caballero, B., Academic Press, New York, pp. 1858–1863, 1998.
- [2] Bailey, A.L., Finglas, P.M., Wright, A.J., and Southon, S.: Thiamin intake, erythrocyte transketolase (EC 2.2.1.1) activity and total erythrocyte thiamin in adolescents. *Br. J. Nutr.*, **72**, 111–125, 1994.
- [3] Cooper, J.R. and Pincus, J.H.: The role of thiamine in nervous tissue. *Neurochem. Res.*, **4**, 223–239, 1979.
- [4] Blass, J.P., Gleason, P., Brush, D., DiPonte, P., and Thaler, H.: Thiamine and Alzheimer's disease. A pilot study. *Arch. Neurol.*, **45**, 833–835, 1988.
- [5] Mastrogicola, F., Bettendorff, L., Grisar, T., and Kish, S.J.: Brain thiamine, its phosphate esters, and its metabolizing enzymes in Alzheimer's disease. *Ann. Neurol.*, **39**, 585–591, 1996.
- [6] Kanofsky, J.D.: Thiamin status and cognitive impairment in

- the elderly. *J. Am. Coll. Nutr.*, **15**, 197–198, 1996.
- [7] Booth, A.A., Khalifah, R.G., Todd, P., and Hudson, B.G.: *In vitro* kinetic studies of formation of antigenic advanced glycation end products (AGEs). Novel inhibition of post-Amadori glycation pathways. *J. Biol. Chem.*, **272**, 5430–5437, 1997.
- [8] Wolff, S.P., Jiang, Z.Y., and Hunt, J.V.: Protein glycation and oxidative stress in diabetes mellitus and ageing. *Free Radic. Biol. Med.*, **10**, 339–352, 1991.
- [9] Jain, S.K., Kannan, K., and Lim, G.: Ketosis (acetoacetate) can generate oxygen radicals and cause increased lipid peroxidation and growth inhibition in human endothelial cells. *Free Radic. Biol. Med.*, **25**, 1083–1088, 1998.
- [10] Higashi-Okai, K., Kanbara, K., Amano, K., Hagiwara, A., Sugita, C., Matsumoto, N., and Okai, Y.: Potent antioxidative and antigenotoxic activity in aqueous extract of Japanese rice bran-association with peroxidase activity. *Phytother. Res.*, **18**, 628–633, 2004.
- [11] Nakano, M.: Assay for superoxide dismutase based on chemiluminescence of luciferin analog. *Methods Enzymol.*, **186**, 227–232, 1990.
- [12] Noda, Y., Kohno, M., Mori, A., and Packer, L.: Automated electron spin resonance free radical detector assays for antioxidant activity in natural extracts. *Methods Enzymol.*, **299**, 28–34, 1999.
- [13] Asakawa, T. and Matsushita, S.: A colorimetric micro-determination of peroxide values utilizing aluminum chloride as the catalyst. *Lipids*, **15**, 965–967, 1980.
- [14] Imada, I., Sato, E.F., Miyamoto, M., Ichimori, Y., Minamiyama, Y., Konaka, R., and Inoue, M.: Analysis of reactive oxygen species generated by neutrophils using a chemiluminescence probe L-012. *Anal. Biochem.*, **271**, 53–58, 1999.
- [15] Onorato, J.M., Jenkins, A.J., Thorpe, S.R., and Baynes, J.W.: Pyridoxamine, an inhibitor of advanced glycation reactions, also inhibits advanced lipoxidation reactions. Mechanism of action of pyridoxamine. *J. Biol. Chem.*, **275**, 21177–21184, 2000.
- [16] Jain, S.K. and Lim, G.: Pyridoxine and pyridoxamine inhibits superoxide radicals and prevents lipid peroxidation, protein glycosylation, and $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity reduction in high glucose-treated human erythrocytes. *Free Radic. Biol. Med.*, **30**, 232–237, 2001.
- [17] Vasani, S., Zhang, X., Zhang, X., Kapurniotu, A., Bernhagen, J., Teichberg, S., Basgen, J., Wagle, D., Shih, D., Terlecky, I., Bucala, R., Cerami, A., Egan, J., and Ulrich, P.: An agent cleaving glucose-derived protein crosslinks *in vitro* and *in vivo*. *Nature*, **382**, 275–278, 1996.
- [18] Wolfenbutter, B.H., Boulanger, C.M., Crijns, F.R., Huijberts, M.S., Poitevin, P., Swennen, G.N., Vasani, S., Egan, J.J., Ulrich, P., Cerami, A., and Levy, B.I.: Breakers of advanced glycation end products restore large artery properties in experimental diabetes. *Proc. Natl. Acad. Sci. U. S. A.*, **95**, 4630–4634, 1998.
- [19] Brown, L.A., Harris, F.L., and Guidot, D.M.: Chronic ethanol ingestion potentiates TNF- α -mediated oxidative stress and apoptosis in rat type II cells. *Am. J. Physiol. Lung Cell Mol. Physiol.*, **281**, L377–386, 2001.
- [20] Hu, M.L., Chen, Y.K., and Lin, Y.F.: The antioxidant and prooxidant activity of some B vitamins and vitamin-like compounds. *Chem. Biol. Interact.*, **97**, 63–73, 1995.
- [21] McPhail, L.C., DeChatelet, L.R., and Johnston, R.B., Jr.: Generation of chemiluminescence by a particulate fraction isolated from human neutrophils. Analysis of molecular events. *J. Clin. Invest.*, **63**, 648–655, 1979.
- [22] Takabe, T., Asami, S., and Akazawa, T.: Glycolate formation catalyzed by spinach leaf transketolase utilizing the superoxide radical. *Biochemistry*, **19**, 3985–3989, 1980.
- [23] Porter, N. and Wujek, D.G.: Autoxidation of polyunsaturated fatty acids, an expanded mechanistic study. *J. Am. Chem. Soc.*, **106**, 2626–2629, 1984.
- [24] Noguchi, T. and Nakano, M.: Effect of ferrous ions on microsomal phospholipid peroxidation and related light emission. *Biochim. Biophys. Acta*, **368**, 446–455, 1974.
- [25] Thomas, M.J., Mehl, K.S., and Pryor, W.A.: The role of the superoxide anion in the xanthine oxidase-induced autoxidation of linoleic acid. *Biochem. Biophys. Res. Commun.*, **83**, 927–932, 1978.