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Inhibition of free radical generation by biotin

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Abstract—To assess the inhibitory effect of biotin on free radical generation, we used a spectrophotometric assay of cytochrome *c* reduction and determined the 2-methyl-6-phenyl-3,7-dihydroimidazo[1,3-*a*]-pyrazin-3-one (CLA)-dependent chemiluminescence response of human neutrophils or a hypoxanthine-xanthine oxidase (XOD) system. In the spectrophotometric assay of cytochrome *c* reduction, superoxide (O_2^-) generation by neutrophils stimulated with *N*-formyl-methionyl-leucyl-phenylalanine (f-MLP) was reduced significantly when biotin was added. In the CLA-dependent chemiluminescence test of neutrophils stimulated by f-MLP, biotin significantly reduced the gneration of free radical species, including O_2^-, in a concentration-dependent manner, the concentration corresponding to 50% inhibition (IC_{50}) of biotin for free radical generation was 1.12×10^{-7} mol. However, biotin did not exert an inhibitory effect on oxidative metabolism by directly scavening superoxide anion, as shown by the study using the hypoxanthine-XOD system.

Key words: biotin, superoxide, neutrophil, chemiluminescence, cytochrome *c* reduction

Biotin (vitamin H; $C_{10}H_{16}N_2O_3S$) is a member of the vitamin B group and was initially isolated from egg yolk as an enzymatic yeast growth factor [1]. It was later confirmed to be identical to coenzyme R (Rhizobium growth factor) [2], and its pharmacological action has been demonstrated to be that of a carbonic acid fixation coenzyme. However, the mechanisms underlying these biochemical effects of biotin are not understood completely.

There have been many reports on vitamins, especially α-tocopherol and ascorbic acid or their related compounds, as radical scavengers [3–8]. In the present study, to determine the effect of biotin on free radical generation, we studied its ability to inhibit CLA*-dependent chemiluminescence induced by human neutrophils or by a hypoxanthine-XOD system, and also performed a spectrophotometric assay to assess cytochrome *c* reduction.

Materials and Methods

Chemicals. An injectable biotin preparation (1.02×10^5, 1.02×10^{-6}) and a placebo (the same solvent) were obtained from the Sansyou Seiyaku Co. Acetazolamido injection solution was obtained from the Nihon Lederle Co., thioctic acid injection solution from the Fujisawa Yakuhin Co. and Conray 400 from the Daiichi Seiyaku Co. f-MLP and horse heart cytochrome *c* (type VI) were purchased from the Sigma Chemical Co. Ficoll 400 was obtained from the Pharmacia Co., and CLA was purchased from Tokyo Kasei Kogyou. Hypoxanthine, XOD, and the other materials used were purchased from Wako Pure Chemical Industries.

Preparation of neutrophil lysates. Heparinized (10 U/mL) venous blood was obtained from one healthy volunteer (a 30-year-old man). Purified preparations of neutrophils were isolated from the blood by dextran sedimentation and the method of Böyum [9] with slight modifications. These leukocytes were suspended in 10 mL of Hanks' balanced salt solution (HBSS) (pH 7.4), which contained 8.0 g NaCl, 0.4 g KCl, 0.14 g $CaCl_2$, 0.2 g $MgSO_4$–$7H_2O$, 0.06 g Na_2HPO_4–$2H_2O$, 0.06 g KH_2PO_4, and 1.0 g glucose in a total volume of 1 L. This leukocyte suspension was loaded onto 15 mL of a Conray 400–Ficoll 400 fluid (pH 7.4 and a density of 1.0785, prepared by mixing of 167 mL of Conray 400 and 72.0 g of Ficoll 400 in a total volume of 1 L, and centrifuged at 400 *g* for 15 min.

The purity of the neutrophils was more than 95% as determined by Wright staining. The viability was more than 99% by the trypan blue dye exclusion test.

Spectrophotometric methods. The standard reaction mixture contained cells (1×10^6), cytochrome *c* (0.1 mM), f-MLP (1×10^{-6} M) and Hank's buffer (pH 7.4) in a total volume of 3.0 mL with or without biotin (4.1×10^{-4} M). The mixture was incubated at 37° for 5 min and chilled immediately to 0°. The reaction mixture was then centrifuged at 250 *g* for 10 min, and the absorbance of the supernatant at 550 nm was measured by spectrophotometry.

Chemiluminescence method. The standard reaction mixture for testing human neutrophils contained cells (1×10^6), f-MLP (1×10^{-6} M), CLA (5×10^{-7} M), and Hanks' buffer (pH 7.4) in a total volume of 2.0 mL with biotin (1×10^{-4} to 1×10^{-7} M), placebo or no additive. Measurement of chemiluminescnece was initiated by the addition of CLA to the standard incubation mixture without f-MLP for 3 min, followed by incubation for a further 1 min after the addition of f-MLP. In addition, CLA was added to the standard mixture without f-MLP and biotin, and after incubation for 3 min, f-MLP was added, followed by biotin.

The experiment, using a hypoxanthine-XOD system, followed the original method of Kimura and Nakano [10]. The reaction mixture contained CLA (1×10^{-5} M), hypoxanthine (5×10^{-5} M), XOD (6.5 mU) and 50 mM Tris–HCl buffer with 0.1 mM EDTA (pH 7.8), in a total volume of 3.0 mL. Measurement of chemiluminescence was initiated by the addition of CLA to the incubation mixture without XOD for 3 min, followed by incubation for a further 3 min after the addition of XOD. Chemiluminescence was measured at 37° using a luminescence reader (Aloka, BLR102).

Statistical analysis. All values are given as mean ± SD. Group means were compared by Student's *t*-test.

Results

Spectrophotometric assay of cytochronme c *reduction.* We initially performed an experiment utilizing cytochrome *c* reduction in order to determine what effect biotin had

* Abbreviations: CLA, 2-methyl-6-phenyl-3,7-dihydro-imidazo[1,2-*a*]pyrazin-3-one; f-MLP, *N*-formyl-methionyl-leucyl-phenylalanine; O_2^-, superoxide; and XOD, xanthine oxidase.

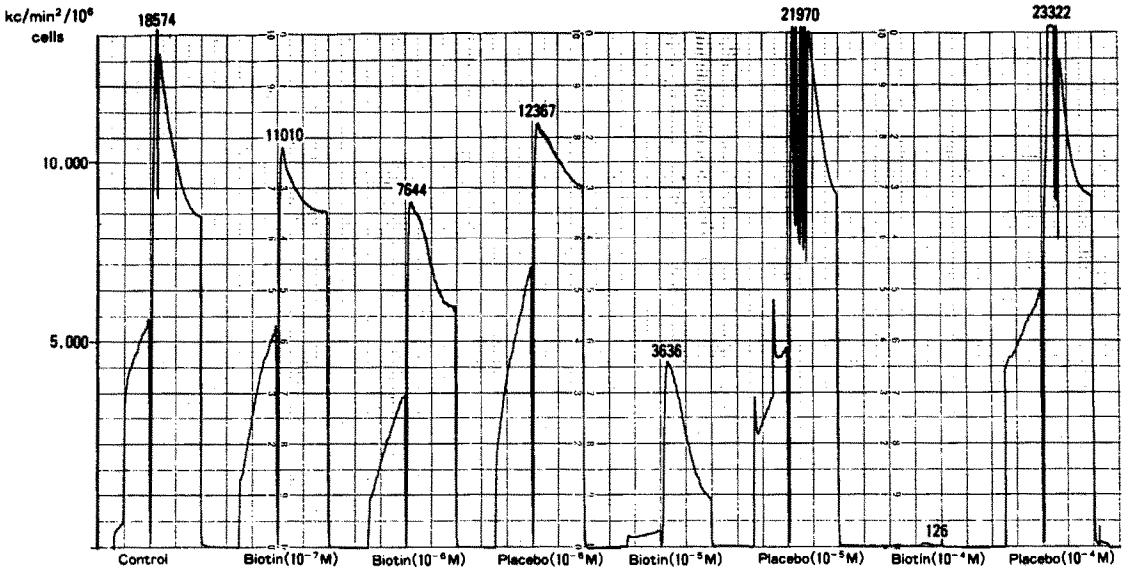

Fig. 1. Measurement of CLA-dependent chemiluminescence activity in the presence of biotin. Measurement of chemiluminescence was initiated by the addition of CLA (5×10^{-7} M) to the standard incubation mixture without f-MLP for 3 min, followed by incubation for a further 1 min after the addition of f-MLP (1×10^{-6} M).

on O_2^- production by neutrophils. There were two biotin groups (A and B) and two non-biotin groups (C and D), and these were either stimulated (A or C) or not stimulated (B or D) with f-MLP. To confirm reproducibility, the identical specimens were measured six times each, and the Δ-index was determined using the following formula:

$$\Delta OB(A - B \text{ or } C - D)/19.1 \times 10^3 \times 1/15 \times 10^6/\text{polymorphonuclear cell (mmol/min)}.$$

The values of cytochrome *c* reduction were 273.00 ± 7.80 mmol/min for the stimulated biotin group (A), 269.83 ± 4.71 mmol/min for the non-stimulated biotin group (B), 264.67 ± 8.73 mmol/min for the stimulated non-biotin group (C), and 281.33 ± 6.22 mmol/min for the non-stimulated non-biotin group (D). The Δ-index for the biotin groups (−3.16 ± 4.71) was significantly lower than that for the non-biotin groups (16.65 ± 6.22) ($P < 0.01$). It was also determined that biotin alone had no effect on absorbance at 550 nm.

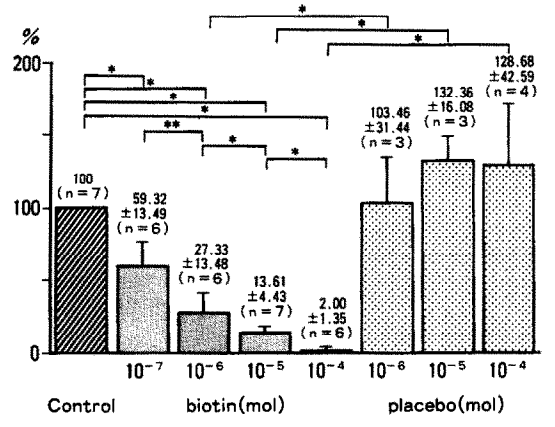

Fig. 2. Effect of biotin (1×10^{-4} to 1×10^{-7} M) on CLA-dependent chemiluminescence of neutrophils stimulated by f-MLP (1×10^{-6} M). The placebo experiments used the same solvent fluids without biotin. Results are the means ± SD of 6–7 experiments (control and biotin) or 3–4 experiments (placebo), and are expressed as a percentage of the control level determined with cells not exposed to any drug (control value: 13,771.38 ± 3,554.55). Key: (*) $P < 0.001$, and (**) $P < 0.01$.

CLA-dependent chemiluminescence of human neutrophils. Next, we investigated the effect of biotin using CLA-dependent chemiluminescence (kc/min²/10^6 cells), a more sensitive method than the spectrophotometric assay. Immediate chemiluminescence was observed when f-MLP was added after incubating the cells and CLA. This chemiluminescence was inhibited when biotin was added in advance, but no such effect was detected when the placebo was added (Fig. 1).

When the control value was set at 100%, the CLA-dependent chemiluminescence activity in the presence of biotin was 59.32 ± 13.49% at 1×10^{-7} mol, 27.33 ± 13.48% at 1×10^{-6} mol, 13.61 ± 4.43% at 1×10^{-5} mol, and 2.00 ± 1.35% at 1×10^{-4} mol. With the placebo, on the other hand, the values were 103.46 ± 31.44% at 1×10^{-6} mol, 132.36 ± 16.08% at 1×10^{-5} mol, and 128.68 × 42.59% at 1×10^{-4} mol. Thus, the percentages were significantly lower in the presence of biotin (Fig. 2), and the concentration corresponding to 50% inhibition (IC_{50}) of biotin was 1.12×10^{-7} mol (Fig. 3). Furthermore, when f-MLP was added after incubation of the cells with CLA, chemiluminescence was still suppressed when biotin was added subsequently.

CLA-dependent chemiluminescence of the hypoxanthine-XOD system. We investigated the oxidant scavenger action of biotin using a hypoxanthine-XOD system, which generates O_2^- chemically. When the control value was set at 100%, the values in the presence of biotin were 91.08 ± 12.53% at 1×10^{-7} mol, 99.95 ± 8.04% at 1×10^{-6} mol, 93.95 ± 9.82% at 1×10^{-5} mol, and 72.53 ± 11.56% at 1×10^{-4} mol. No significant difference from the control value was found.

Discussion

In this study, we investigated the effect of biotin on free

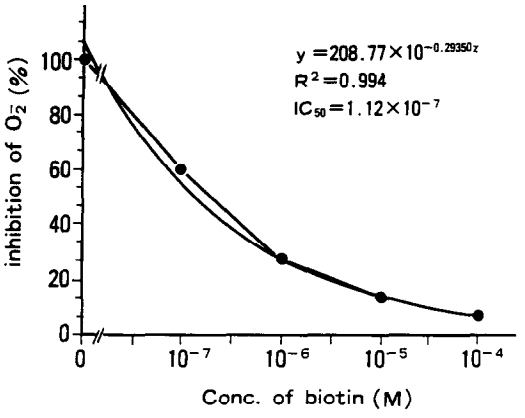

Fig. 3. Concentration-dependent inhibition of neutrophilic CLA-dependent chemiluminescence by biotin (1×10^{-4} to 1×10^{-7} M) following stimnulation by f-MLP (1×10^{-6} M). The concentration of biotin required for 50% inhibition (IC_{50}) was about 1.12×10^{-7} mol.

radical generation by three different methods: cytochrome *c* reduction, the CLA-dependent chemiluminescence response of human neutrophils, and the hypoxanthine-XOD system. Many free radical species including O_2^- have been detected in the CLA-dependent chemiluminescence test of neutrophil activity [11], whereas O_2^- alone is specifically detected when cytochrome *c* reduction occurs [12]. In the hypoxanthine-XOD system, O_2^- is generated through the chemical reaction of hypoxanthine with XOD [13]. In the spectrophotometric assay of cytochrome *c* reduction, O_2^- generation from neutrophils stimulated by f-MLP was reduced significantly by the addition of biotin. In addition, biotin significantly reduced the generation of free radical species (including O_2^-) in a concentration-dependent manner in the CLA-dependent chemiluminescnce test of neutrophils stimulated by f-MLP (Fig. 2). This test showed that the inhibitory effect of biotin on free radical generation was quite strong (IC_{50}: 1.12×10^{-7} mol) (Fig. 3). Moreover, the inhibitory effect was similar even when biotin was added after the addition of f-MLP.

To determine whether biotin acts as a scavenger in the same manner as α-tocopherol or ascorbic acid, we conducted a study using the hypoxanthine-XOD O_2^- generation metabolism by directly scavening superoxide anion. These findings confirmed that biotin has no direct scavenging effect or f-MLP antagonist effect. Thus, it was concluded that biotin may modulate the signal transduction pathway which activates NADPH oxidase.

Recently, the signal transduction pathways for O_2^- generation by neutrophils were investigated [14], and it is suggested that biotin affects these pathways. The receptors are coupled to G-proteins that activate phospholipase C to release diacylglycerol (which remains in the membrane to act as a substrate for protein kinase C) and inositol-1,4,5-triphosphate (which enters the cytoplasm where it releases Ca^{2+} from intracellular stores).

In place of biotin, we also conducted similar experiments with sodium acetazolamide ($C_4N_4NaO_3S_2$) and thioctic acid ($C_8H_{14}O_2S_2$), which have analogous structures and two S-radicals, but these drugs had no effect on the inhibition of O_2^- generation by activated neutrophils (data not shown). Further work is now necessary to establish (a) how biotin affects protein kinase C or Ca^{2+} mobilization in neutrophils, (b) whether any of the effects of biotin are due to inhibition of neutrophil O_2^- generation, and (c) whether biotin has any clinical effects on diseases suggested to be free radical-related.

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First Department of Internal Medicine
Gunma University School of Medicine
Maebashi 371, Japan

TETSURO SEKIGUCHI*
TAKEAKI NAGAMINE

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* Corresponding author: Dr. Tetsuro Sekiguchi, 3-39-15 Showa machi, Maebashi, Gunma 371, Japan. Tel. 272-(31)-7221; FAX 272-(33)-2872.