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Prooxidant action of maltol: Role of transition metals in the generation of reactive oxygen species and enhanced formation of 8-hydroxy-2′-deoxyguanosine formation in DNA

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Abstract

Maltol (3-hydroxy-2-methyl-4-pyrone) produced reactive oxygen species as a complex with transition metals. Maltol/iron complex inactivated aconitase the most sensitive enzyme to oxidative stress. The inactivation of aconitase was iron-dependent, and prevented by TEMPOL, a scavenger of reactive oxygen species, suggesting that the maltol/iron-mediated generation of superoxide anion is responsible for the inactivation of aconitase. Addition of maltol effectively enhanced the ascorbate/copper-mediated formation of 8-hydroxy-2′-deoxyguanosine in DNA. Oxidation of ascorbic acid by $CuSO_4$ was effectively stimulated by addition of maltol, and the enhanced oxidation rate was markedly inhibited by the addition of catalase and superoxide dismutase. These results suggest that maltol can stimulate the copper reduction coupled with the oxidation of ascorbate, resulting in the production of superoxide radical which in turn converts to hydrogen peroxide and hydroxyl radical. Cytotoxic effect of maltol can be explained by its prooxidant properties: maltol/transition metal complex generates reactive oxygen species causing the inactivation of aconitase and the production of hydroxyl radical causing the formation of DNA base adduct.

Introduction

Maltol (3-hydroxy-2-methyl-4-pyrone), a degradation product of carbohydrates, is found in coffee, chicory, roasted malt and caramelized foods (Gralla *et al*. 1969; Ito 1977; Bjeldanes & Chew 1979), and is widely used as a flavoring agent to give 'freshly baked flavor' to bread and cakes (Bjeldanes & Chew 1979). The hydroxy pyrone structure in maltol shows a potent metal-chelating activity (Yasumoto *et al*. 2004): some vanadium-containing maltol complexes exhibit various biological activities (Thompson *et al*. 2003). Several studies showed the maltol-dependent enhancement of aluminum toxicity: maltol-mediated enhancement of aluminum toxicity in neuronal cells (Savory *et al*. 1993) can be explained by effective absorption of aluminum/maltol complex at neutral pH (Crapper-McLachlan 1986). Our previous report also showed that maltol/aluminum complex effectively induced an apoptosis of PC12D cells (Tsubouchi *et al*. 2001). However, information about the toxicity of maltol itself is quite limited (Langui *et al*. 1990). In this paper we describe the maltol/metal-mediated generation of reactive oxygen species. Production of reactive oxygen species was demonstrated by the inactivation of aconitase (EC 4.2.1.3) the sensitive enzyme to active oxygen. Maltol further stimulated the ascorbate/copper-dependent formation of 8-hydroxy-2′-deoxyguanosine in DNA, which is an indicator of hydroxyl radical and singlet oxygen. Cytotoxic action of maltol may be explained by the prooxidant properties of chelation complexes with metals.

Materials and methods

Materials

Maltol, dimethylpyrone, threo-Ds-isocitrate, catalase (specific activity of 2000–6000 μmol/min/mg protein), superoxide dismutase (specific activity of 2000–5000 μmol/min/mg), chelex and TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Yeast NADP-isocitrate dehydrogenase (specific activity of 30 μmol/min/mg) was a product of Oriental Yeast Co. (Tokyo, Japan). All chemicals were of analytical grade, and enzymes used in the present study were essentially homogeneous preparations as judged by SDS-polyacrylamide gel electrophoresis. Baker's yeast was purchased locally.

Determination of aconitase activity

Commercial baker's yeast was permeabilized with toluene (Murakami *et al.* 1980). The cells (10 mg/ml) were incubated with maltol and $FeSO_4$ in 20 mM potassium phosphate buffer (pH 7.1), and were used for determination of enzyme activities. Aconitase activity was determined spectrophotometrically by the increase in the absorbance at 340 nm with coupling method using NADP-isocitrate dehydrogenase. Reaction mixture of 1 ml contained 5 mM citrate, 2 mM $MgCl_2$, 0.25 mM NADP, 0.05 unit of purified NADP-isocitrate dehydrogenase from yeast and 50 mM Tris–HCl buffer (pH 7.1). Reaction was started by the addition of cell extract. Statistical analyses were performed by Student's *t*-test.

Quantitation of 8-hydroxy-2′-deoxgyguanosine in calf thymus DNA treated with maltol and copper

Calf thymus DNA was treated with ascorbic acid and $CuSO_4$ in the presence of maltol. The reaction mixture of 4 ml contained 100 μg of calf thymus DNA, 0.1 mM ascorbic acid, 0.1 mM $CuSO_4$ and various concentrations of maltol in 10 mM Tris–HCl buffer (pH 7.4). The mixture was incubated at 37 °C for 60 min. Aliquots of 10 μl were analyzed by 0.8% agarose gel electrophoresis, and stained with ethidium bromide. The remainder was hydrolyzed and used for the determination of 8-hydroxy-2′-deoxyguanosine according to the method reported previously (Yoshino *et al.* 1999). Deoxyribonucleosides and 8-hydroxy-2′-deoxyguanosine were detected using an ESA electrochemical detector (Homma *et al.* 1994). Unmodified nucleosides were detected by UV absorption. The amount of 8-OHdG present in the DNA samples was calculated by measuring the area of the peaks obtained from both electrochemical and UV traces and comparing those obtained from DNA samples with those obtained from the standards. Statistical analysis was performed by Student's *t*-test.

Oxidation of ascorbic acid by copper

The sample of 1 ml contained 10 mM Tris–HCl buffer (pH 7.4), 2 μM $CuSO_4$, 0.1 mM ascorbic acid, 0.1 mg/ml Chelex in the absence and presence of 0.1 mM maltol and additives. The mixture was incubated at 37 °C, and the absorbance at 263 nm was recorded. In order to examine the role of reactive oxygen species, 25 μg/ml catalase and 50 μg/ml superoxide dismutase were included in the mixture.

Results

We examined the effect of maltol on the activity of aconitase the most sensitive enzyme to reactive oxygen species (Gardner & Fridovich 1992, Murakami & Yoshino 1997, Gardner 2002) with the permeabilized yeast cells. Addition of maltol with iron caused an effective inactivation of aconitase, but dimethyl pyrone did not show any effect (Figure 1a). The activities of aldolase and glyceraldehyde 3-phosphate dehydrogenase were not at all inactivated by maltol (data not shown). Pretreatment of cells with TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl), a scavenger of reactive oxygen species, protected aconitase from the maltol-mediated inactivation effectively (Figure 1b). These results suggest that maltol/iron complex produced reactive oxygen species causing the inactivation of aconitase.

When calf thymus DNA was treated with ascorbic acid in the presence of $CuSO_4$, 8-OHdG was effectively formed (Yoshino *et al.* 1999). We examined the effect of maltol on the copper-dependent 8-OHdG formation. Addition of maltol

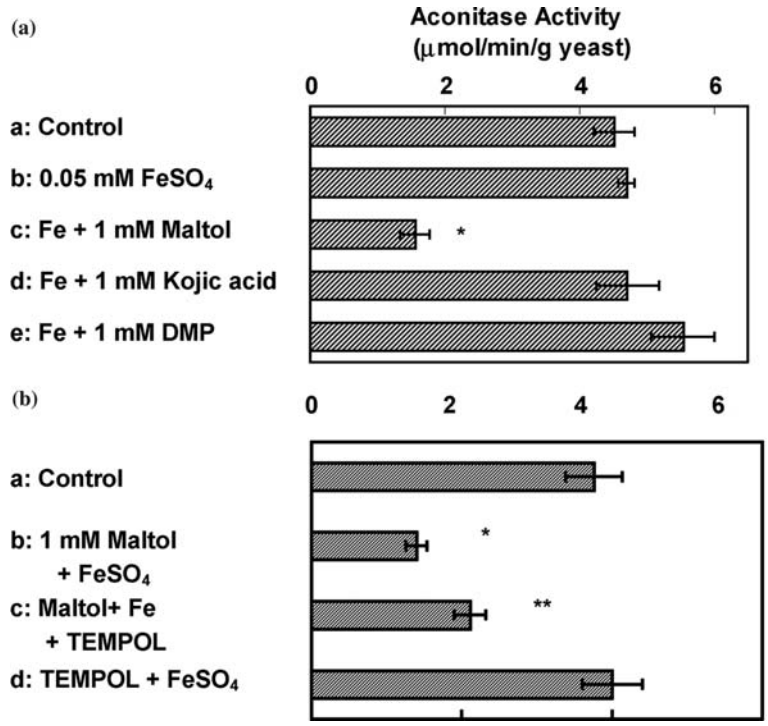

Figure 1. Effect of maltol, kojic acid and dimethylpyrone compounds on the aconitase activity in permeabilized yeast cells. (a) Inactivation of aconitase by maltol. Baker's yeast cells (10 mg/ml) permeabilized with toluene (Murakami *et al*. 1980) were incubated with 1 mM maltol, kojic acid and dimethylpyrone derivatives in the presence of 0.05 mM $FeSO_4$ for 10 min, and the enzyme activities were determined. (a) Control (no addition); (b) 0.05 mM $FeSO_4$ added; (c) maltol with $FeSO_4$ added; (d) kojic acid with $FeSO_4$ added; (e) dimethylpyrone with $FeSO_4$ added. (b) Effect of TEMPOL on the maltol/iron-mediated inactivation of aconitase. TEMPOL of 2.5 mM was added to permeabilized yeast, followed by addition of maltol/iron complex. (a) Control (0.05 mM $FeSO_4$ added); (b) 1 mM maltol with 0.05 mM $FeSO_4$ added; (c) maltol with TEMPOL plus $FeSO_4$ added; (d) TEMPOL plus $FeSO_4$ added. Asterisks indicate a significant difference in the aconitase activity: $^{*}P < 0.001$ (between the maltol/Fe group and the control), $^{**}P < 0.05$ (between the maltol/Fe/TEMPOL group and the maltol/Fe group).

increased the formation of 8-OHdG about 20–30% (Figure 2). Addition of catalase completely inhibited the formation of 8-OHdG, indicating that maltol enhanced the formation of hydroxyl radical resulting from hydrogen peroxide.

Formation of hydroxyl radical may be dependent on the stimulation of ascorbate-mediated formation of reduced copper. Therefore, we examined the effect of maltol on the oxidation of ascorbate coupled with the reduction of copper. Figure 3 shows that maltol effectively enhanced the copper-dependent oxidation of ascorbic acid, and further addition of superoxide dismutase and catalase effectively inhibited the oxidation of ascorbic acid. These results suggest that maltol effectively stimulated the reduction of copper coupled with oxidation of ascorbate, resulting in the enhanced reduction of molecular oxygen by cuprous ion and then in the formation of superoxide radical that converts to hydrogen peroxide.

Discussion

Maltol is widely used as a flavoring agent to bread and cake (Bjeldanes & Chew 1979), and shows various biological activities due to the potent metal-chelating activity resulting from its hydroxy pyrone structure (Yasumoto *et al*. 2004). For example, vanadyl maltolate compounds with insulin-like activity are applied to therapy for diabetes (Thompson *et al*. 2003), and ferric trimaltol can correct iron deficiency anemia by enhanced absorption in alimental canal (Harvey *et al*. 1998). On the other hand, some metal/maltol chelate complexes show various cytotoxic effects: maltol/aluminum complex deteriorates aluminum toxicity

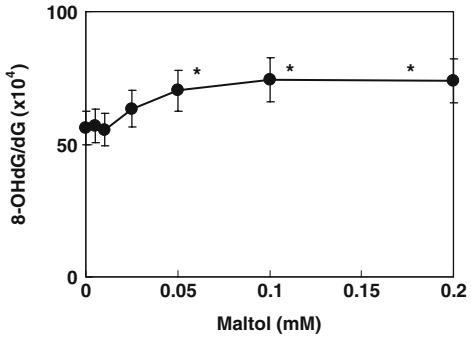

Figure 2. Effect of maltol on the ascorbate/copper-dependent formation of 8-hydrdoxy-2′-deoxyguanosine in DNA. Calf thymus DNA was treated with 0.1 mM ascorbate plus 0.1 mM $CuSO_4$ in the absence and presence of the indicated concentrations of maltol, and 8-hydrdoxy-2′-deoxyguanosine formed was determined by HPLC-ECD method after nuclease treatment. Each point represents the mean ± SD of three independent experiments. Asterisks indicate a significant difference in the 8-OhdG/dG ratio between the control and the maltol/Fe-treated groups ($P < 0.01$).

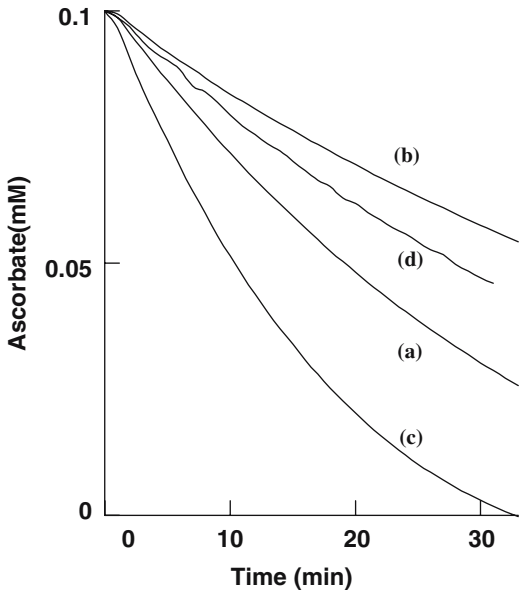

Figure 3. Effect of maltol on the copper-dependent oxidation of ascorbic acid. The mixture of 1 ml contained 0.1 mM ascorbic acid, 2 μM $CuSO_4$ in the absence and presence of 0.1 mM maltol with or without superoxide dismutase (50 μg/ml) and catalase (25 μg/ml). (a) Control (2 μM $CuSO_4$ only); (b) catalase + superoxide dismutase added; (c) 0.1 mM maltol added; (d) maltol added with catalase and superoxide dismutase. One experiment typical of four is shown.

in neuronal tissues (Savory *et al*. 1993), and induces apoptosis of cells (Tsubouchi *et al*. 2001). These findings suggest that maltol/metal complex can produce reactive oxygen species. The present work showed that maltol could act as a prooxidant by generating reactive oxygen species, which was demonstrated by the inactivation of aconitase, the most sensitive to reactive oxygen species (Gardner & Fridovich 1992, Murakami & Yoshino 1997, Gardner 2002), and by the protective action of TEMPOL. Prooxidant property of maltol was further demonstrated by the stimulation of the ascorbate/copper-mediated oxidative modification of DNA base.

Hydroxyketone compounds such as maltol, deferiprone and mimosine are capable of chelating metal ion, in pariticular ferric ion with high affinity (Moridani & O'Brien 2001). The oxygen atom in the conjugated carbonyl group in maltol can convert to oxygen centred radical by the reaction with ferric ion. The centred oxygen radical would react with O_2 to form the superoxide radical (Fiore *et al*. 2004), which inactivates aconitase by oxidizing the prosthetic iron–sulfur cluster $[4Fe–4S]^{2+}$ at the active site, resulting in the formation of the inactive $[3Fe–4S]^{1+}$ enzymes and then in the release of iron(II) from the enzyme active sites. Ferrous ion is responsible for further reduction of molecular oxygen to superoxide radical, which in turn generates hydrogen peroxide. Finally, hydrogen peroxide, by interacting with the reduced transition metal such as Fe^{2+}, produces hydroxyl radical by means of Fenton reaction. Hydroxyl radical the most potent oxidant participates in anti-microbial and cytotoxic effects. Aconitase is a sensitive indicator to reactive oxygen species, and maltol-mediated inactivation of the enzyme further participates in the enhanced production of hydroxyl radical (Vásquez-Vivar *et al*. 2000). Maltol further can enhance the ascorbate/copper-mediated formation of 8-hydroxy-2′-deoxyguanosine in DNA. Stimulation of the DNA base adduct formation can be explained by enhancement of ascorbate-dependent copper reduction. Increase in cuprous ion may be responsible for the formation of DNA base adduct by increasing hydroxyl radical formation.

Our previous work showed that maltol can act as an antioxidant by inhibiting the iron-mediated lipid peroxidation (Murakami *et al*. 2001). Discrepancy between the previous results and the present ones may be explained by the decreased perferryl ion: formation of ferric trimaltol complex

inhibits the formation of perferryl ion, which initiates lipid peroxidation (Miller & Aust 1989). Thus, maltol can act as an antioxidant under certain conditions. Maltol-mediated stimulation of transition metal reduction shows a principal role in the production of reactive oxygen species causing apoptosis-inducing, anti-microbial and anti-tumoral effects, and will be applicable for various chemotherapeutic uses.

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