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Prooxidant action of xanthurenic acid and quinoline compounds: role of transition metals in the generation of reactive oxygen species and enhanced formation of 8-hydroxy-2′-deoxyguanosine in DNA†

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Abstract

Xanthurenic acid, a product of tryptophan–NAD pathway, and quinoline compounds produced reactive oxygen species as a complex with iron. Aconitase, the most sensitive enzyme to oxidative stress was inactivated effectively by xanthurenic acid and to a lesser extent by 8-quinolinol in the presence of ferrous sulfate. The inactivation of aconitase was iron-dependent, and was prevented by TEMPOL, a scavenger of reactive oxygen species, suggesting that reduced iron bound to xanthurenic acid or 8-quinolinol can activate oxygen molecule to form superoxide radical. However, kynurenic acid and quinaldic acid without 8-hydroxyl group did not produce reactive oxygen species. Of the quinoline compounds tested, xanthurenic acid and 8-quinolinol with 8-hydroxyl group stimulated the autooxidation of ferrous ion, but kynurenic acid and quinaldic acid did not affect the oxidation of ferrous ion. Hydroxyl group at 8-positions of quinoline compounds was essential for the binding of iron causing the generation of reactive oxygen species. 8-Quinolinol effectively enhanced the ascorbate/copper-mediated formation of 8-hydroxy-2′-deoxyguanosine in DNA, suggesting the quinolinol/copper-dependent stimulation hydroxyl radical formation. Xanthurenic acid and 8-quinolinol as the metal–chelate complexes can show various cytotoxic effects by generating reactive oxygen species through the ferrous or cuprous ion-dependent activation of oxygen molecule.

Introduction

Xanthurenic acid with a quinoline structure is one of the products of tryptophan metabolism, and is formed through the kynurenine pathway. Tryptophan loading and vitamin B_6 deficiency stimulate the formation of xanthurenic acid and tryptophan metabolites in kynurenine pathway (Forrest *et al.* 2004), and the increased xanthurenic acid level is an indicator of vitamin B_6 deficiency (Adams *et al.* 1976). Several reports were presented concerning the tryptophan loading-mediated oxidative stress and the role of xanthurenic acid in the pathogenesis of diabetic states (Rogers & Mohan 1994) and senile cataract (Malina & Martin 1996; Malina 1999). Recently xanthurenic acid was shown to be photochemically active, and to photooxidize lens proteins in eye (Roberts *et al.* 2000): the oxidizable properties of xanthurenic acid were discussed to be related to the cataractogenesis (Roberts *et al.* 2001; Thiagarajan *et al.* 2002). Xanthurenic acid with quinoline ring acts as a potent iron chelator (O'Sullivan & Smithers 1979), and some metal–chelate complexes are capable of forming reactive

† This paper is dedicated to centennial of the birthday of the late Professor Emeritus Yahito Kotake, a pioneer of the xanthurenic acid research.

oxygen species (Welch *et al*. 2002), we here analyzed the xanthurenic acid/iron-mediated generation of reactive oxygen species, which was proved by the inactivation of aconitase (EC 4.2.1.3) the sensitive enzyme to active oxygen. Prevention of this inactivation by TEMPOL the scavenger of reactive oxygen species confirmed the xanthurenic acid/iron-mediated generation of reactive oxygen species. Xanthurenic acid 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 xanthurenic acid may be explained by the prooxidant properties of chelate complexes with metals.

Materials and methods

Chemicals

Xanthurenic acid, kynurenic acid, 8-hydroxyquinoline (8-quinollinol), quinaldic acid, bathophenanthroline disulfonate, citrate, NADP and TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) were products of Sigma-Aldrich-Japan (Tokyo, Japan). 2-Morpholinoethanesulfonic acid was a product of Dojindo Co. (Kumamoto, Japan). Chemicals used in the present study were of reagent grade. NADP-isocitrate dehydrogenase (specific activity above 30 μmol/min per mg protein) was a product of Oriental Yeast Co. (Tokyo, Japan). 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 xanthurenic acid or various quinoline compounds, 0.05 mM $FeSO_4$, and 1 mM sodium azide in 20 mM potassium phosphate buffer (pH 7.1) for 10 min, 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 addition of the cell. Statistical analyses were performed by Student's *t*-test.

Autooxidation of Fe^{2+} ion

Interaction of xanthurenic acid and quinoline compounds with iron was evaluated by the effect of these compounds on the rate of autooxidation of Fe^{2+} ion as described previously (Yoshino & Murakami 1998). The samples of 2 ml contained 10 mM Tris–HCl (pH 7.1), 0.1 mM $FeSO_4$ and quinoline compounds. The reaction was started by addition of $FeSO_4$. Aliquots of 0.2 ml were mixed with 0.1 ml of 1 mM bathophenanthroline disulfonate at appropriate intervals, and the absorbance at 540 nm was measured.

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

Calf thymus DNA was treated with ascorbic acid and $CuCl_2$ in the presence of 8-quinolinol. The reaction mixture of 4 ml contained 100 μg of calf thymus DNA, 0.1 mM ascorbic acid, 0.1 mM $CuCl_2$ and various concentrations of 8-quinolinol 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 (8-OHdG) according to the method reported previously (Yoshino *et al*. 1999). Deoxyribonucleosides and 8-hydroxy-2′-deoxyguanosine were detected using an ESA Electrochemical detector. 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.

Results

We examined the effect of xanthurenic acid and its related quinoline compounds (Figure 1) on the

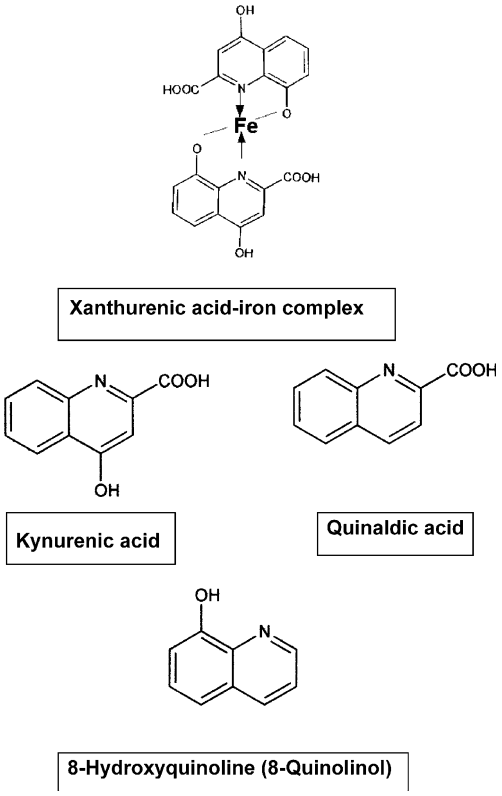

Figure 1. Structure of xanthurenic acid and its related quinoline compounds.

activity of aconitase the most sensitive enzyme to reactive oxygen species (Gardner & Fridovich 1992; Murakami & Yoshino 1997; Gardner 2002) using the permeabilized yeast cells. Xanthurenic acid inactivated aconitase in the presence of $FeSO_4$, but did not affect glyceraldehyde 3-phosphate dehydrogenase. Kynurenic acid did not show any inactivating effect on aconitase and glyceraldehyde 3-phosphate dehydrogenase (Figure 2). Structural specificity of the quinoline compounds causing the inactivation of aconitase was examined. Of the quinoline compounds tested, xanthurenic acid and 8-quinolinol caused an effective and less potent inactivation of the enzyme, respectively, but other quinoline compounds did not show any inactivating effect (Figure 3a). EDTA, a typical metal chelator, also did not inactivate aconitase (data not shown). Addition of TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl), a scavenger of reactive oxygen species, protected aconitase against the xanthurenic acid-mediated inactivation effectively (Figure 3b). These results suggest that the inactivation of aconitase was ascribed to the

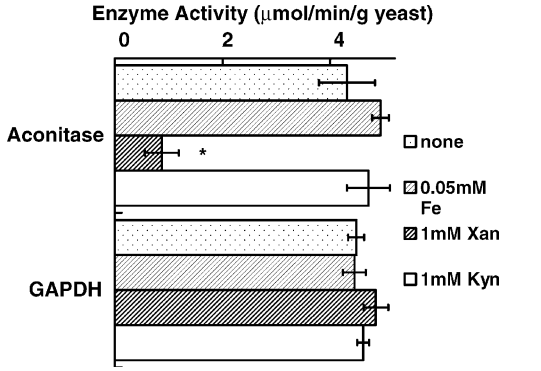

Figure 2. Effect of xanthurenic acid on the activities of aconitase and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in permeabilized yeast cells. Baker's yeast cells (10 mg/ml) permeabilized with toluene (Murakami *et al.* 1980) were incubated with 1 mM xanthurenic acid (XA) or kynurenic acid (KA) in the presence of 0.05 mM $FeSO_4$ and 1 mM sodium azide for 10 min, and the enzyme activities were determined. Each data represents mean ± SD of three different determinations. Asterisk indicates a significant difference between the control group without xanthurenic acid and the xanthurenic acid-treated group ($P < 0.001$).

xanthurenic acid/iron complex-mediated formation of reactive oxygen species. We further examined the concentration dependency of xanthurenic acid on the inactivation of aconitase. The concentration of xanthurenic acid required for 50% inactivation was about 0.15–0.2 mM (Figure 4).

Oxidation and reduction of transition metals are closely related to the antioxidant and prooxidant action. We now examined the effect of xanthurenic acid and its derivatives on the autooxidation of Fe^{2+} ion. Xanthurenic acid stimulated the autooxidation of ferrous ion markedly, and 8-quinolinol also enhanced the Fe^{2+} autooxidation to a lesser extent (Figure 5). Kynurenic acid showed only a little stimulating effect on the Fe^{2+} oxidation, and quinaldic acid did not show any effect.

When calf thymus DNA was treated with ascorbic acid in the presence of $CuCl_2$, 8-OHdG was effectively formed (Yoshino *et al.* 1999). We tried to examine the effect of quinoline compounds on the copper-dependent 8-OHdG formation, but could not analyze the prooxidant action of xanthurenic acid on DNA because xanthurenic acid interfered the determination of 8-OHdG in the HPLC-ECD system. We thus examined the effect of 8-quinolinol on the formation of 8-OHdG. Formation of 8-OHdG increased with the increase in the 8-quinolinol, and rose to a value 1.5 times as

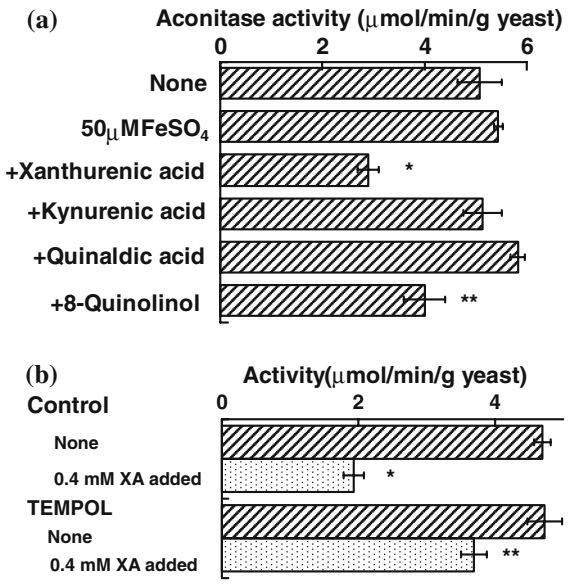

Figure 3. Effect of quinoline compounds on the aconitase activity in permeabilized yeast cells. Experimental conditions were similar to those of Figure 2 except that various quinoline compounds at 0.5 mM were included. (a) Effect of quinoline compounds on the aconitase activity. (b) Effect of TEMPOL on the aconitase activity in the presence of xanthurenic acid/iron. Prior to the addition of xanthurenic acid/$FeSO_4$ to permeabilized yeast, 1 mM TEMPOL was added, and aconitase activity was determined 1 h after the treatment. Control, without treatment by TEMPOL; TEMPOL group, treated with TEMPOL prior to the addition of xanthurenic acid/$FeSO_4$. (XA, xanthurenic acid). Each data represents mean ± SD of three different determinations. Asterisks indicate a significant difference; *, $P < 0.001$ between the control group and the xanthurenic acid-treated group; **, $P < 0.01$ between the xanthurenic acid-treated group without and with TEMPOL.

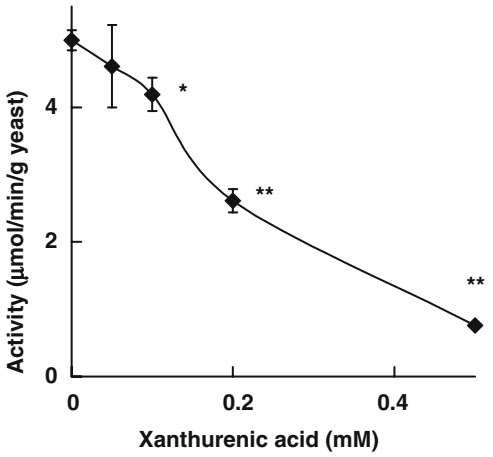

Figure 4. Concentration-dependent inactivation of aconitase by xanthurenic acid in permeabilized yeast. Experimental conditions were similar to those of Figure 2 except that xanthurenic acid concentrations were varied. Each data represents mean ± SD of three different determinations. Asterisks indicate significant differences between the control group without xanthurenic acid and the xanthurenic acid-treated groups. *, $P < 0.05$; **, $P < 0.001$

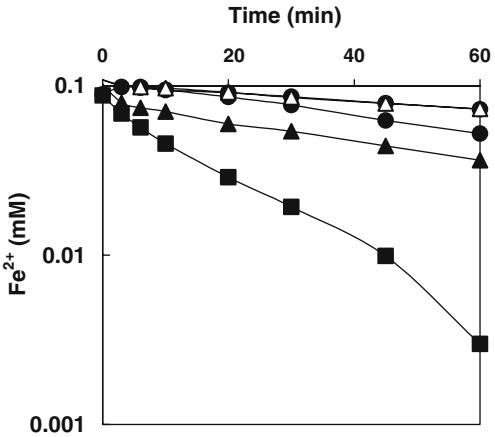

Figure 5. Effect of xanthurenic acid and quinoline compounds on the autooxidation of Fe^{2+} ion. Iron oxidation of followed by determining the Fe^{2+} ion concentrations with bathophenanthroline sidulfonate as described previously (Yoshino & Murakami 1998). Closed circle, none; open triangle, quinaldic acid; closed circle, kynurenic acid; closed triangle, 8-quinolinol; closed square, xanthurenic acid.

much by addition of 0.1 mM 8-quinolinol. However, further increase in 8-quinolinol rather inhibited the formation of 8-OHdG (Figure 6). Catalase completely inhibited the formation of 8-OHdG, indicating that 8-quinolinol enhanced the formation of hydroxyl radical resulting from hydrogen peroxide (data not shown).

Discussion

Xanthurenic acid is related to various pathological conditions (Rogers & Mohan 1994; Malina & Martin 1996; Malina 1999; Roberts *et al*. 2000, 2001; Thiagarajan *et al*. 2002), although the biological function of this compound remains obscure. Xanthurenic acid production is increased by tryptophan administration (Forrest *et al*. 2004) and vitamin B_6 deficiency in humans and animals (Adams *et al*. 1976). In particular, xanthurenic acid is formed largely depending on the level of vitamin B_6, which acts as the pyridoxal coenzyme of tryptophan–NAD metabolic pathway. Vitamin B_6 deficiency markedly decreases the activity of kynureninase (EC 3.7.1.3), the pyridoxal dependent enzyme catalyzing the main

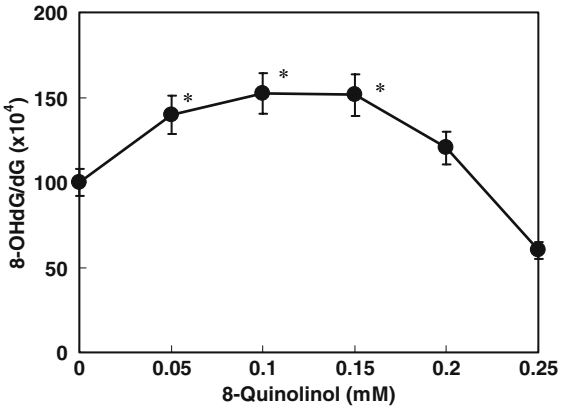

Figure 6. Effect of 8-quinolinol on the ascorbate/copper-dependent formation of 8-hydroxy-2′-deoxyguanosine in DNA. Calf thymus DNA was treated with 0.1 mM ascorbate plus 0.1 mM $CuCl_2$ in the absence and presence of the indicated concentrations of 8-quinolinol, and 8-hydrdoxy-2′-deoxyguanosine formed was determined by HPLC-ECD method after nuclease treatment (Yoshino *et al.* 1999). Each data represents mean ± SD of three different determinations. Asterisks indicate significant differences between the control group without 8-quinolinol and the 8-quinolinol-added groups. *, $P < 0.05$.

route of tryptophan–NAD pathway, but affects the activity of kynurenine aminotransferse that forms xanthurenic acid only a little. Decreased kynureninase activity with a little change in the kynurenine aminotransferase activity increases the production of xanthurenic acid, an excellent indicator of vitamin B_6 deficiency (Ogasawara *et al.* 1962). Early studies on xanthurenic acid and vitamin B_6 deficiency were focused on the abnormalities in carbohydrate metabolism (Rogers & Mohan 1994). Furthermore, xanthurenic acid was shown to provoke formation of unfolded proteins in endoplasmic reticulum of the lens epithelial cells (Malina 1999). Recent studies revealed that xanthurenic acid acts as a photosensitizer and generates superoxide and singlet oxygen upon irradiation (Roberts *et al.* 2000). Photooxidation and polymerization by xanthurenic acid of lens proteins are related to the age-dependent cataractogenesis (Roberts *et al.* 2001).

Of the quinoline compounds tested, xanthurenic acid and 8-quinolinol with 8-hydroxyl group produced reactive oxygen species in the presence of iron, and showed a stimulating effect on the autooxidation of ferrous ion. Furthermore, the 8-quinolinol enhanced the formation of 8-hydroxy-2′-deoxyguanosine in DNA. The present findings indicate that quinoline compounds with 8-hydroxyl group such as xanthurenic acid can act as a prooxidant. On the contrary, xanthurenic acid sometimes acts as an antioxidant: our previous study showed that xanthurenic acid inhibits the iron-mediated lipid peroxidation, and protects NADP-isocitrate dehydrogenase by chelating iron and copper (Murakami & Yoshino 2001). However, formation of metal–chelate complex modifying the oxidation–reduction potential of metal ion is responsible for the generation of reactive oxygen species (Welch *et al.* 2002). Metal-dependent generation of reactive oxygen species, thus, depends on the formation of metal–chelate complex and the activation of oxygen molecule by reduced metal ion (Welch *et al.* 2002). Binding of metals to quinoline compounds requires 8-hydroxyl group, and the compounds with 8-hydroxyl group show high stability constants for metals (O'Sullivan & Smithers 1979). No production of reactive oxygen species by kynurenic acid and quinaldic acid may be ascribed to the inability of these quinoline compounds without 8-hydroxyl group to bind metals.

Xanthurenic acid and 8-hydroxyquinoline bind ferric ion at the 8-hydroxyl group and the nitrogen atom of the quinoline moiety, resulting in the enhancement of the autooxidation of ferrous ion to ferric ion. Oxygen molecules accept one electron from ferrous ion to form superoxide radical, 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. Superoxide radical in turn generates hydrogen peroxide through the dismutation reaction. Finally, hydrogen peroxide, by interacting with the reduced transition metal such as Fe^{2+}, produces hydroxyl radical the most potent oxidant by means of Fenton reaction. Aconitase is a sensitive indicator to reactive oxygen species, and xanthurenic acid-mediated inactivation of the enzyme further participates in the enhanced production of hydroxyl radical (Vásquez-Vivar *et al.* 2000).

Stimulating effect of 8-hydroxyquinoline on the ascorbate/copper-dependent formation of 8-hydroxy-2′-deoxyguanosine is also related to the metal-binding properties of this compound. Ascorbic acid reduces copper ion located at poly G and GC sequences in DNA, and cuprous ion

formed further activates oxygen molecule to form superoxide radical and hydrogen peroxide as discussed above. Hydroxyl radical generated from hydrogen peroxide can react with guanine base form 8-hydroxy-2′-deoxyguanosine (Yoshino *et al.* 1999). Our present results are well agreed with the previous findings that iron/8-hydroxyquinoline complex causes DNA strand breaks and lipid peroxidation in cultured lung cells (Leanderson & Tagesson 1996). Oxidative DNA damage was stimulated by 8-quinolinol below 0.15 mM, but was rather inhibited by the increase in this compound in the presence of 0.2 mM $CuCl_2$ (Figure 6), suggesting that the oxidative action of quinoline compounds depends on the formation of redox-active metal complex at the molar ratio of 8-quinolinol to copper below 2. Excess quinoline compounds may form redox-inactive complexes, which cannot react with ascorbic acid. Similar redox properties of metal–tropolone complex were recently reported. Tropolone, a typical iron chelator forms a complex with metals at different molar ratio: tropolone can stimulate the oxidative DNA damage at the ratio of tropolone/iron lower than 3, but shows a preventive effect on the iron-mediated DNA damage at the molar ratio of tropolone to iron more than 3 (Doulias *et al.* 2005). We also confirmed the generation of reactive oxygen species from the tropolone/iron complex at the molar ratio of 1 (Murakami *et al.* 2005).

Tryptophan loading-mediated oxidative stress has been explained by the action of the increased 3-hydroxykynurenine and 3-hydroxyanthranilic acid with prooxidant nature (Forrest *et al.* 2004). However, as demonstrated in this paper, xanthurenic acid also could act as a prooxidant by formation of redox-active chelate complex: xanthurenic acid–metal–chelate complex at the concentration below 0.15 mM initiated cellular oxidative damage by producing reactive oxygen species. Furthermore, xanthurenic acid was demonstrated to act as an apoptosis-inducing metabolite: treatment of vascular smooth muscle and lens epithelial cells with 0.1–0.2 mM xanthurenic acid causes apoptotic cell death (Malina *et al.* 2001, 2002). The xanthurenic acid concentration necessary for the apoptosis induction is comparable to that required for oxidative formation of DNA base adduct and inactivation of aconitase, indicating that xanthurenic/metal-mediated generation of reactive oxygen species may cause the pathogenesis of various diseases accompanied with the increase in xanthurenic acid.

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