All-*trans* Arachidonic acid generates reactive oxygen species via xanthine dehydrogenase/xanthine oxidase interconversion in the rat liver cytosol *in vitro*

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We previously reported that the all-*cis* isomer of arachidonic acid, the most naturally occurring isoform of this fatty acid, reduced cuprous copper ion-induced conversion of xanthine dehydrogenase into its reactive oxygen species generating form, xanthine oxidase. In the present study, the effects of all-*trans* isomer of arachidonic acid, in comparison with *cis* isomer of arachidonic acid, on the xanthine dehydrogenase/xanthine oxidase interconversion were explored. *cis* isomer of arachidonic acid alone did not have any significant effect on the activities of xanthine dehydrogenase and xanthine oxidase, but it inhibited the cuprous copper ion-induced conversion of xanthine dehydrogenase to xanthine oxidase in rat liver cytosol *in vitro*. In contrast, *trans* isomer of arachidonic acid elicited an increase in xanthine oxidase activity concomitant with a decrease in xanthine dehydrogenase activity, and further potentiated the cuprous copper ion-induced xanthine dehydrogenase/ xanthine oxidase interconversion. In primary rat hepatocyte cultures, *trans* isomer of arachidonic acid increased 2',7'-dichlorofluorescein-fluorescence intensity in the cytosolic fraction from 2',7'-dichlorodihydrofluorescein, an indicator of reactive oxygen species generation. The pretreatment of allopurinol, an xanthine oxidase inhibitor, diminished the *trans* isomer of arachidonic acid-induced increase in the 2',7'-dichlorofluorescein-fluorescence intensity, indicating the role of xanthine dehydrogenase/xanthine oxidase in mediating *trans* isomer of arachidonic acid-induced reactive oxygen species generation. These observations suggest that, in contrast to all-*cis* arachidonic acid, all-*trans* arachidonic acid has the potential to enhance reactive oxygen species generation via xanthine dehydrogenase/xanthine oxidase interconversion in the liver cytosol *in vitro*.

Key Words: all-*trans* arachidonic acid, all-*cis* arachidonic acid, xanthine oxidase, xanthine dehydrogenase, reactive oxygen species

L iver diseases, in particular ischemia-reperfusion injury, are associated with oxidative damage of the biological macromolecules. The actual generator of reactive oxygen species (ROS) in ischemic and reperfused liver tissue is still unclear, but a xanthine dehydrogenase/xanthine oxidase (XD/XO)-mediated process is considered to be an important source of ROS during ischemia-reperfusion. Ghoneim *et al.*,[1] Fernandez *et al.*[2] and Curek *et al.*[3] have reported the conversion of XD/XO to the XO type which generates ROS under ischemic conditions.

To date, various factors that elicit the conversion of XD to XO

have been found *in vitro*. Della Corte and Stirpe[4] and Kaminski and Jezewska[5] have reported that cupric copper ion (Cu²⁺) can convert XD to XO. We have shown that this metal ion can induce the conversion of XD to XO in rabbit liver cytosol at several micromolar concentration ranges.[6] We have also reported that all-*cis* arachidonic acid (CAA), the most naturally existing isoform of this fatty acid, has the potential to inhibit the Cu²⁺-induced conversion of XD to XO.[7]

In recent years, the role of the naturally occurring *cis-trans* lipid geometry has attracted attention. *trans* Lipids are studied as dietary components that can exert harmful effects on human health. Despite numerous epidemiological and experimental feeding studies,[8–12] the molecular targets of *trans* fatty acids in mediating their pathological functions are not fully elucidated. In this letter, we report the effects of all-*trans* AA (TAA), in comparison with CAA, on the conversion of XD to XO in the presence or absence of Cu²⁺ in the rat liver cytosol *in vitro*.

Materials and Methods

Materials. CAA, xanthine, NAD⁺ and allopurinol were purchased from Sigma Chemical Co. (St. Louis, MO). 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR) and 4',6-diamino-2-phenylindole (DAPI) was from Merck (San Diego, CA). Other reagents were of analytical grade.

Preparation of TAA. TAA was synthesized following the procedure of Anagnostopoulos *et al.*,[13] with slight modifications. Briefly, a 3.0 ml solution of CAA (10 mg) in isopropanol was placed in a quartz photochemical reactor and bubbled with nitrogen for 20 min. 2-Mercaptoethanol in a 50% mole equivalent (15 μl) with respect to CAA was added and the mixture was exposed to a low-pressure mercury lamp (5.5 W) at 25°C.[14] After 40 min photolysis, the mixture was concentrated under a vacuum, and the crude TAA was then purified by preparative Ag/silica gel TLC with n-pentane-chloroform-acetone (3:9:8, v/v/v). The fractions were detected by spraying a portion of the plate with cerium ammonium sulfate/ammonium molybdate reagent. The TAA fraction was scraped and extracted from silica gel using chloroform. This TLC procedure was repeated for further purification (yield; about 10%). CAA and the chemically synthesized product TAA

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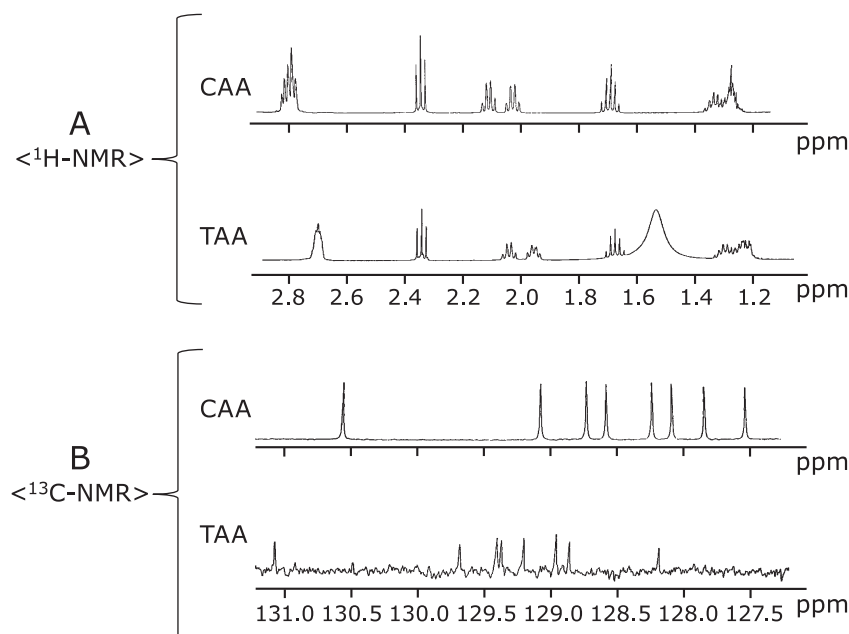J. Clin. Biochem. Nutr. | July 2012 | vol. 51 | no. 1 | 55–60

Fig. 1. ¹H (A) and ¹³C (B) NMR spectra for CAA and TAA. CAA, all-*cis* arachidonic acid; TAA, all-*trans* arachidonic acid.

were identified using ^{1}H and ^{13}C NMR analysis (Fig. 1), which were similar to the previously reported NMR spectra.[13] A 40-min photolysis was suitable for formation of the all-trans isomer of AA (purity; >97%).

Animal experiments. Animal experiments were performed according to the experimental animal guidelines of the Osaka University of Pharmaceutical Sciences and of the Japanese Ministry of Education, Culture, Sports, Science and Technology.

Measurement of XD and XO activities in rat liver cytosol. The partially purified enzyme fraction from the rat liver cytosol was prepared according to the method reported in our previous paper.[15–17] The activities of XD and XO were assayed by monitoring uric acid formation using the HPLC method. XO activity was determined by measuring uric acid formation in the absence of NAD$^+$. XD activity was calculated by subtracting the amount of uric acid in the absence of NAD$^+$ (XO activity) from that in the presence of NAD$^+$ (XO plus XD activity).

Detection of ROS in primary rat hepatocyte cultures. Primary rat hepatocytes were isolated and cultured following the protocol described in our previous paper.[18] The production of intracellular ROS was measured by using the oxidation-sensitive fluorescent dye DCFH-DA.[19,20] The hepatocytes prepared from rat liver were seeded in flexiPERM put on slides at 25,000 cells/ 1 ml/5 cm^2 well. One day after seeding, the medium was changed to the serum free medium. The rat primary hepatocytes were incubated with CAA and TAA for 5 min, then DCFH-DA was added at a final concentration of 5 μM. After a 15 min-incubation, ROS were measured using a confocal laser scanning microscope. The intensity of production was recorded after excitation at 495 nm and emission at 530 nm. Data were collected from at least 10 random sections per sample.

Protein determination. Protein concentration was determined by the method of Lowry *et al.*[21] using bovine serum albumin as the standard.

Statistics. Results are the means ± SE. The significance of the differences between two groups was assessed by the *t* test, and differences between multiple groups were assessed by one-way analysis of variance (ANOVA) followed by Scheffe's multiple range test. *p* values less than 0.05 were considered significant.

Results

Fig. 2 shows the effects of various concentrations of CAA and TAA on the activities of XD and XO in the cytosolic fraction from the rat liver. Under basal conditions, XO activity represented about 15–20% of the total XD plus XO activity, implying that, in normal physiological conditions, most XD/XO exists as the XD type. CAA at concentrations up to 100 μM had no significant effect on the XD and XO activities (Fig. 2A). On the other hand, as shown in Fig. 2B, TAA at concentrations ranging from 25 to 100 μM elicited increased XO activity together with decreased XD activity. The effects of TAA were statistically significant at concentrations of 75 and 100 μM. These results imply that TAA, but not CAA, has the potential to enhance the conversion of XD to XO. Furthermore, both elaidic and linolelaidic acids, the *trans* isomers of oleic and linoleic acids, were without significant effects on the XD/XO interconversion [None: XO activity, 0.91 ± 0.08, XD activity, 4.86 ± 0.31; 100 μM elaidic acid: XO activity, 1.02 ± 0.09, XD activity, 4.70 ± 0.35; 100 μM linolelaidic acid: XO activity, 1.07 ± 0.08, XD activity, 4.91 ± 0.32 (uric acid formation, nmol/min/mg, $n = 3$)]. These findings suggest that, of the *trans* fatty acids, the interconversion of XD to XO is relatively specific for TAA.

In mammalian species, XD is converted into XO via reversible sulfhydryl oxidation, or irreversible proteolysis of XD.[22,23] Linas *et al.*[24] concluded that the conversion of XD into XO in renal ischemia is mainly reversible. McKelvey *et al.*[25] have also suggested that irreversible XO may be mostly derived from a reversible XO intermediate, rather than directly from XD. We have previously shown that the XO formed by the treatment of copper, zinc and selenium ions, peroxynitrite and disulfide *S*-monoxides is completely reconverted to XD by dithiothreitol (DTT), a sulfhydryl reagent.[6,15,17] Thus, in an attempt to clarify the mechanism by which TAA converts XD into XO, we examined the effect of DTT on XO converted from XD by TAA (Fig. 3). DTT (0.5 mM) alone did not show any effect on the XO and XD activities. DTT (0.1 and 0.5 mM) added after a 10-min preincubation with the TAA (100 μM) reconverted the preformed XO into XD. The results of this study may indicate that TAA converts XD
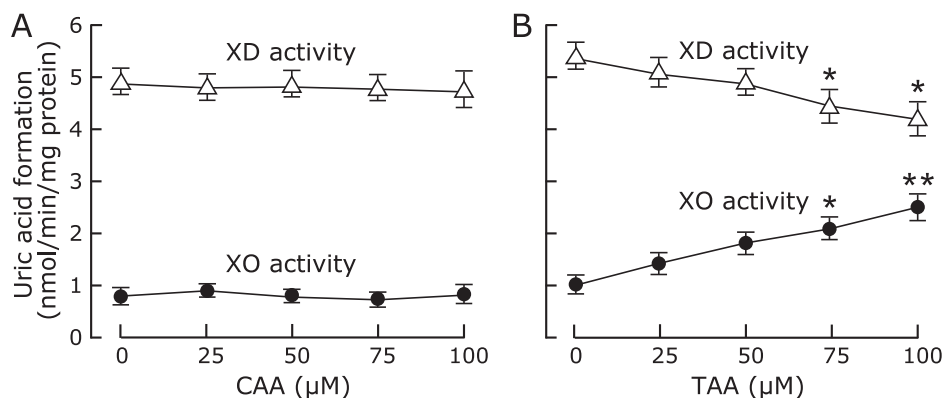
Fig. 2. Effects of CAA (A) and TAA (B) on the activities of XD and XO in the cytosolic fraction from the rat liver. The cytosolic fraction from the rat liver was incubated with xanthine alone or xanthine plus NAD$^+$ in the presence or absence of CAA or TAA. XO activity = uric acid formation by xanthine alone. XD activity = uric acid formation by xanthine plus NAD$^+$-xanthine alone. Results are mean values ± SE ($n = 4$). *$p<0.05$, **$p<0.01$; compared with the corresponding values in the absence of CAA or TAA. CAA, all-*cis* arachidonic acid; TAA, all-*trans* arachidonic acid; XO, xanthine oxidase; XD, xanthine dehydrogenase.
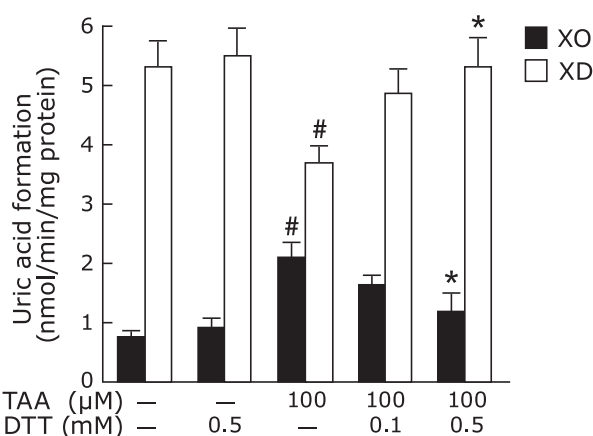

Fig. 3. Effects of DTT posttreatment on the TAA-induced conversion of XD to XO in the cytosolic fraction from the rat liver. The cytosolic fraction was preincubated with xanthine alone or xanthine plus NAD$^+$ in the presence or absence of TAA for 10 min. Dithiothreitol (DTT) was added to the incubation mixture at $t = 0$ min. XO activity = uric acid formation by xanthine alone. XD activity = uric acid formation by xanthine plus NAD$^+$-xanthine alone. Results are mean values ± SE ($n = 3$). #$p<0.05$; compared with the corresponding values in the absence of TAA or DTT. *$p<0.05$; compared with the corresponding values in the presence of TAA alone. CAA, all-*cis* arachidonic acid; TAA, all-*trans* arachidonic acid; XO, xanthine oxidase; XD, xanthine dehydrogenase.

reversibly into XO, and that this effect is due to the modification of XD sulfhydryl groups.

The results shown in Fig. 2 indicated that TAA can convert XD into the ROS-producing XO in the partially purified cytosolic fraction from the rat liver. Therefore, we next investigated whether TAA increases the generation of ROS in rat hepatocytes via the XD/XO pathway (Fig. 4). As shown in Fig. 4A, the treatment of rat hepatocytes with H$_2$O$_2$ (10 and 50 μM) generated more green coloring in cytoplasm than in the control, validating usefulness of DCF-fluorescence method for detecting ROS in viable cells. Treatment of the hepatocytes with TAA (50 and 100 μM) but not CAA (100 μM) stimulated ROS formation as measured by an increase in DCF fluorescence (green) (Fig. 4A). Although an XO inhibitor, allopurinol (0.5 mM), alone did not show any effect (data not shown), this drug diminished the TAA

(100 μM)-induced DCF fluorescence (Fig. 4B). In addition, to confirm that TAA activates DCFH only indirectly to its fluorescent product DCF, in the cytosol-free system the generation of DCF from DCFH (10 μM) in the presence or absence of TAA was monitored by a MTP-880 spectrofluorometer (Corona Electric Co. Ltd., Ibaragi, Japan) with excitation wavelength set at 490 nm and emission at 530 nm. We prepared DCFH from DCFH-DA by NaOH hydrolysis.[26] As a positive control, we could detect DCF fluorescence from DCFH by ultraviolet irradiation. TAA at a concentration of 100 μM did not cause any considerable change of the fluorescence compared to the control [None, 1484 ± 32; 100 μM TAA, 1451 ± 41 (fluorescence intensity, $n = 6$)]. This finding therefore indicates that TAA does not have the power to generate DCF directly from DCFH. The results of Fig. 4 thus indicate that TAA stimulates ROS generation in the cells through the XD/XO-mediated pathway.

To date, the factor that converts XD to XO during certain diseases such as ischemia-reperfusion injury has not been fully determined. However, Della Corte and Stirpe,[4] Kaminski and Jezewska,[5] and Sakuma *et al*.[6] have reported that Cu^{2+} can convert XD to XO in the rabbit and rat liver cytosol at several micromolar concentration ranges. As Tipton and Cook[27] and Muller *et al*.[28] have reported that the total level of Cu in human or rat liver is about 140 μM, it is possible that Cu^{2+} may be able to induce the conversion of XD to XO *in vivo* provided that a significant amount of this metal is free for interaction with the enzyme under certain disease states. The fact that Cu^{2+} is mobilized following ischemia, as observed by Chevion *et al*.[29] may support the importance of this metal ion in the conversion of XD to XO under such situations. We previously reported that CAA could inhibit the Cu^{2+}-induced conversion of XD to XO in rabbit liver cytosol.[7] In the present study therefore, the effects of TAA in comparison with CAA on the Cu^{2+}-induced conversion of XD to XO in rat liver cytosol were examined (Fig. 5). Cu^{2+} (0.2 μM) alone increased XO activity and decreased XD activity significantly. CAA at concentrations of 25 and 50 μM decreased the Cu^{2+}-induced conversion of XD to XO with statistical significance (Fig. 5A). This effect of CAA was the same as the result reported previously by us.[7] Interestingly, as shown in Fig. 5B, TAA statistically potentiated the Cu^{2+}-induced conversion of XD to XO at concentrations of 25 and 50 μM. As shown in Fig. 2B, TAA alone at concentrations up to 50 μM had no significant effect on both XD and XO activities, and subsequently it may be possible that low concentrations of TAA and Cu^{2+} cooperatively convert XD to XO in the rat liver cytosol.
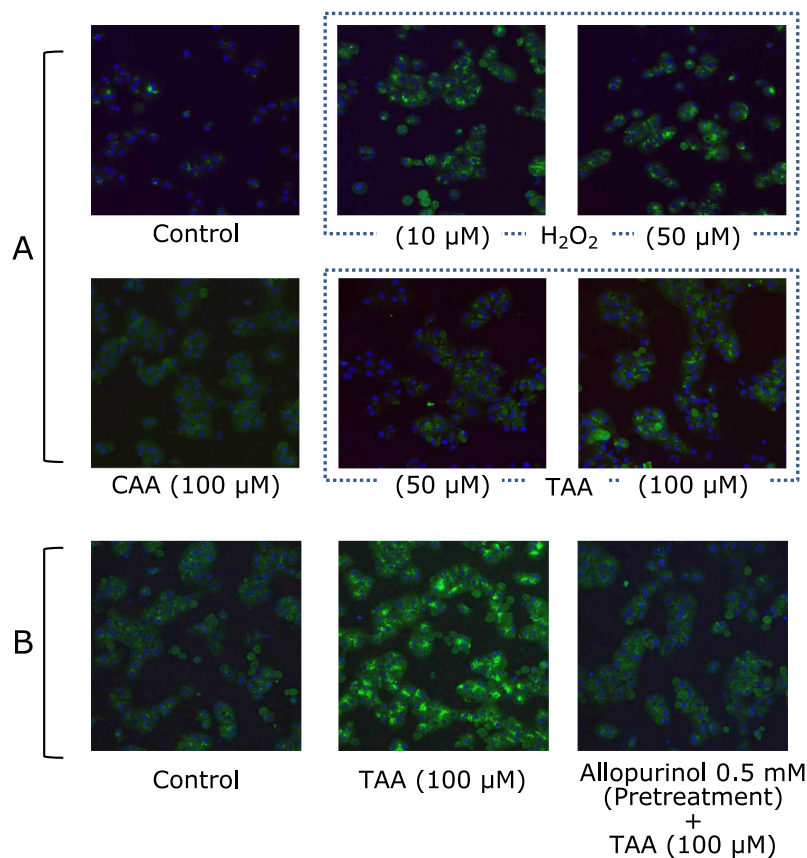
Fig. 4. Effects of TAA on the ROS-induced oxidation of DCFH in rat primary hepatocyte cultures. A: Primary rat hepatocytes (25,000 cells/1 ml/5 cm^2 well) in flexiPERM put on slides were incubated with H$_2$O$_2$ (10 and 50 μM), CAA (100 μM) or TAA (50 and 100 μM) for 5 min, and then DCFH-DA was added at a final concentration of 5 μM. After a 15 min-incubation, ROS were measured using a confocal laser scanning microscope. B: In the case of experiments using an XO inhibitor, allopurinol (0.5 mM), the primary rat hepatocytes were preincubated for 5 min with this drug before addition of TAA. The intensity of the production was recorded after excitation at 495 nm and emission at 530 nm, using appropriate software. Data were collected from at least 10 random sections per sample. The data are representative of 5–6 experiments. CAA, all-*cis* arachidonic acid; TAA, all-*trans* arachidonic acid; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; ROS, reactive oxygen species.
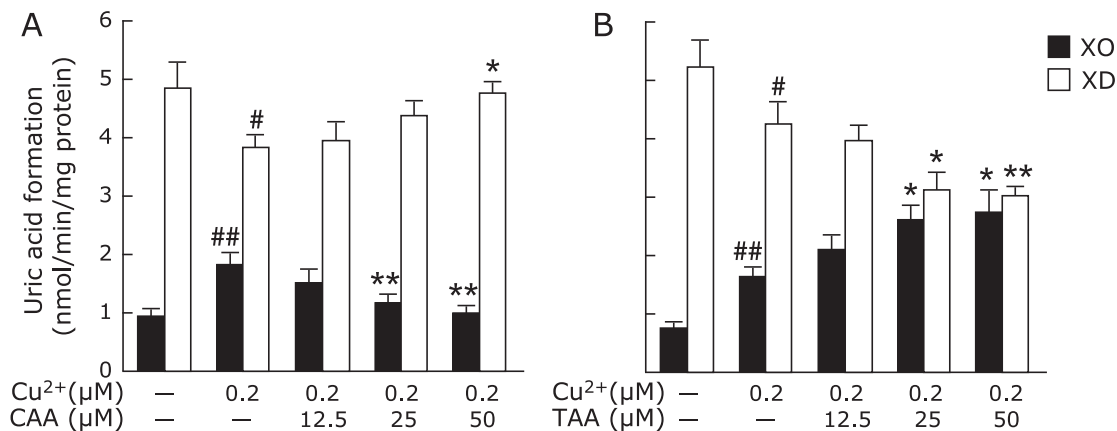

Fig. 5. Effects of CAA (A) and TAA (B) in the presence of Cu^{2+} on the activities of XD and XO in the cytosolic fraction from the rat liver. The cytosolic fraction from the rat liver was incubated with xanthine alone or xanthine plus NAD$^+$ in the presence or absence of CAA or TAA. XO activity = uric acid formation by xanthine alone. XD activity = uric acid formation by xanthine plus NAD$^+$-xanthine alone. Results are mean values ± SE (n = 5). #$p<0.05$, ##$p<0.01$; compared with the corresponding value in the absence of Cu^{2+}, CAA or TAA. *$p<0.01$; compared with the corresponding values in the presence of Cu^{2+} alone. CAA, all-*cis* arachidonic acid; TAA, all-*trans* arachidonic acid; XO, xanthine oxidase; XD, xanthine dehydrogenase.

Discussion

The present study showed for the first time that TAA causes an induction of XO from XD in the presence or absence of Cu^{2+}, and formation of ROS in the liver, which was not predictable from the behavior of CAA reported by us.[7]

The molecular mechanism of the interconversion of XD/XO is still unclear. It is an important issue whether the conversion was a cause for or a result of ROS produced by the TAA administration. Because cyclooxygenase (COX) produce ROS by metabolizing AA and resulted ROS may induce the XD/XO interconversion. But, in the present study, we used the partially purified cytosolic fraction from the rat liver for investigations of Figs. 2, 3 and 5. COX exists in the membrane fraction (especially microsomes), and so it may be difficult to consider TAA metabolism by the COX in the present studies. Furthermore, Balazy and Chemtob[30] have shown that the addition of one TAA isomer, 5E-AA, to the endothelial cells from human coronary and pulmonary arteries generated significant amounts of *trans* isomers of prostaglandin (PG) F2α and PGE2, only when an inducible type of COX, COX-2, was generated by phorbol 12-myristate 13-acetate. Kooli *et al.*[31] further reported that a TAA isomer, 8E-AA stimulation of porcine brain microvascular endothelium cells did not elicit PG production. Because the cytosolic fraction used in the present study was prepared without any stimulus, a possible contaminant might be a constitutive type of COX, COX-1 that does not metabolize TAA into PGs. Furthermore, Fig. 2(A) clearly showed that *cis* arachidonic acid, the main substrate for COX did not have any effect on the XD/XO interconversion. Therefore, it may be difficult to show that TAA makes ROS via COX pathway in the present study.

There is also a possibility that TAA makes ROS directly, and resulted ROS may induce the XD/XO interconversion. However, in the present study, we investigated the effect of TAA on the formation of DCF from DCFH in the cytosol-free system; TAA did not cause any appreciable change of the fluorescence compared to the control. This means that not only TAA makes ROS in the hepatocytes as shown in Fig. 4, but also TAA does not directly generate ROS in the present assay conditions. In addition, Sargis and Subbaiah[32] reported that low-density lipoprotein and phosphatidylcholine liposomes containing *trans* isomers of linoleic acid were more resistant to oxidation than *cis* linoleic acid. Furthermore, Zaima *et al.*[33] reported that *trans* isomers of eicosapentaenoic acid were more stable to oxidation induced by radical generators in organic and in aqueous solution, and were less suitable as a substrate for 15-lipoxygenase oxidation, than *cis* eicosapentaenoic acid.

These findings therefore support the idea that TAA converts XD into XO, which in turn forms ROS in rat liver cytosol *in vitro*.

The differences between TAA and CAA in the XD/XO interconversion shown in Figs. 2 and 5 could be due to the unique geometry of the polyunsaturated molecules. Indeed, the presence of *trans* double bonds is known to confer a more linear molecular shape compared to the *cis* configuration. So, it could be possible that TAA snakes and goes deep into certain site determining XD/XO interconversion, and interacts with and converts XD type to XO type by certain mechanism.

Although most naturally occurring unsaturated fatty acids have only *cis* double bonds, *trans* geometric isomers can be formed during hydrogenation and heat treatment, such as deodorization and deep frying. *trans* Isomers of oleic and linoleic acid, elaidic and linolelaidic acids, are found in several foods including infant formulas, shortenings and vegetable oils. However, TAA must be present in several foods, because *trans* isomerization of CAA also occurs during heat treatment.[34,35] Therefore, the present study suggests that certain harmful effects of TAA in the body are partially due to the ROS formation via XD/XO interconversion, and at the very least TAA and CAA appear to be interesting molecules for investigating the molecular mechanism of XD/XO interconversion.

Abbreviations

CAA	all-*cis* arachidonic acid
Cu^{2+}	cupric copper ion
COX	cyclooxygenase
DAPI	4',6-diamino-2-phenylindole
DCFH-DA	2',7'-Dichlorodihydrofluorescein diacetate
DTT	dithiothreitol
PG	prostaglandin
ROS	reactive oxygen species
TAA	all-*trans* arachidonic acid
XD	xanthine dehydrogenase
XO	xanthine oxidase

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