

Protective Effects of Organic Acids against Xanthine/Xanthine Oxidase-Induced Cell Death by Reducing the Intracellular Level of Hydrogen Peroxide

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ABSTRACT | Enhanced production of superoxide is considered to play a pivotal role in the pathogenesis of various chronic diseases. In the present study, we examined the toxic effects of superoxide and hydrogen peroxide (H_2O_2) produced by xanthine (XA) plus xanthine oxidase (XO), and the protective effects of various organic acids against them by use of a cellular model of COS7 cells, an African green monkey cell line. Here, we report that superoxide and H_2O_2 generated by XA/XO triggered cell death associated with the increase in the intracellular level of H_2O_2 . The reactive oxygen species (ROS) levels were measured by use of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) and a multi-well fluorescence spectrophotometer. XA/XO induced an ROS burst before initiating the loss of cell viability. Catalase and *N*-acetylcysteine protected the cells from the XA/XO-induced cell death, indicating that the effector of the cell death was indeed H_2O_2 . Further, we found that organic acids involved in aerobic energy metabolism, such as pyruvate, oxaloacetate, and α -ketoglutarate, had significant protective effects against the cells death by reducing the levels of H_2O_2 . These organic acids all shared a common chemical structure, i.e., that of α -keto acid, which directly reacted with H_2O_2 . In contrast, other organic acids, such as lactate, succinate, fumarate, and malate, which do not have the α -keto acid structure, but may produce it by dehydrogenase systems, did not efficiently protect the cells, suggesting that this structure was essential for the protective action of organic acids against oxidative stress.

KEYWORDS | Hydrogen peroxide; α -Ketoglutarate; Oxaloacetate; Pyruvate; Reactive oxygen species; Superoxide; Xanthine oxidase

ABBREVIATIONS | AKG, α -ketoglutarate; CA, citrate; CAT, catalase; DCF, dichlorofluorescein; DCFH-DA, 2',7'-Dichlorodihydrofluorescein diacetate; ETC, electron transport chain; FA, fumarate; GPx, glutathione peroxidase; GSH, reduced form of glutathione; KCI, Krebs cycle intermediate; LA, lactate; MA, malate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; OAA, oxaloacetate; PA, pyruvate; PBS, Ca^{2+} - and Mg^{2+} -free phosphate-buffered saline; ROS, reactive oxygen species; SA, succinate; SD, standard deviation; SOD, superoxide dismutase; XA, xanthine; XO, xanthine oxidase

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1. INTRODUCTION

Krebs cycle intermediates (KCI) not only regulate energy metabolism but also are involved in various aspects of biology and medicine [1–3]. Recent reports suggest that KCIs activate specific signaling transduction pathways and exert various biological actions such as neuroprotection, anti-inflammation, osteogenesis, and anti-aging [4, 5]. For example, external supplementation with pyruvate (PA), oxaloacetate (OAA), α -ketoglutarate (AKG), malic acid (MA), or fumarate (FA), but not lactic acid (LA), succinate (SA), citrate (CA), or isocitrate, significantly extends the lifespan of *Caenorhabditis elegans* by activating various transcriptional factor-dependent pathways [6–8].

Energy metabolism and ROS production are closely linked with each other. Superoxide is generated by the electron transport chain (ETC) during aerobic metabolism. The free radical theory of ageing (FRTA) states that organisms age due to the buildup of free radicals over time because these atoms or molecules with an unpaired electron in their outermost shell are unstable and damage cells, thus contributing to aging [9, 10]. Oxidative damage is the most common form of damage initiated by superoxide, which is a byproduct of intracellular processes such as the ETC [11, 12] as well as other oxidases, including NADPH oxidases and xanthine oxidase (XO). While superoxide undergoes auto-dismutation, the enzyme superoxide dismutase (SOD) dramatically accelerates the dismutation of superoxide to form hydrogen peroxide (H₂O₂) [13, 14]. The production of intracellular ROS mostly occurs in the mitochondria

through the ETC, linking the FRTA with mitochondria [15, 16]. In turn, the most powerful reductive power by NADP(H) and reduced form of glutathione (GSH) are highly dependent on aerobic metabolism in mitochondria. In addition, KCIs, which are intermediates of aerobic metabolism in mitochondria, such as PA, OAA, and AKG, are effective antioxidants [15, 16].

The goal of this study was to clarify the mechanism of xanthine (XA)/XO-induced cell death and the protective effects by endogenous organic acids. We found that the α -keto acid group-containing KCIs (PA, OAA, and AKG) could protect cells against XA/XO-induced toxicity, possibly through their direct interaction with H₂O₂ derived from the XA/XO system, thereby resulting in decreased H₂O₂ levels inside the cells.

2. MATERIALS and METHODS

2.1. Chemicals

All the organic acids of the KCIs, XA, XO, catalase (CAT), *N*-acetyl cysteine (NAC), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Wako (Tokyo, Japan). Stock solutions of KCIs (sodium salt, 100 mM, or 1000 mM) were prepared in Ca²⁺- and Mg²⁺-free phosphate-buffered saline (Invitrogen, Carlsbad, CA, USA). 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma-Aldrich, St. Louis, MO, USA) stock solution was prepared in 10 mM dimethyl sulfoxide and used at 10 μ M in the culture medium.

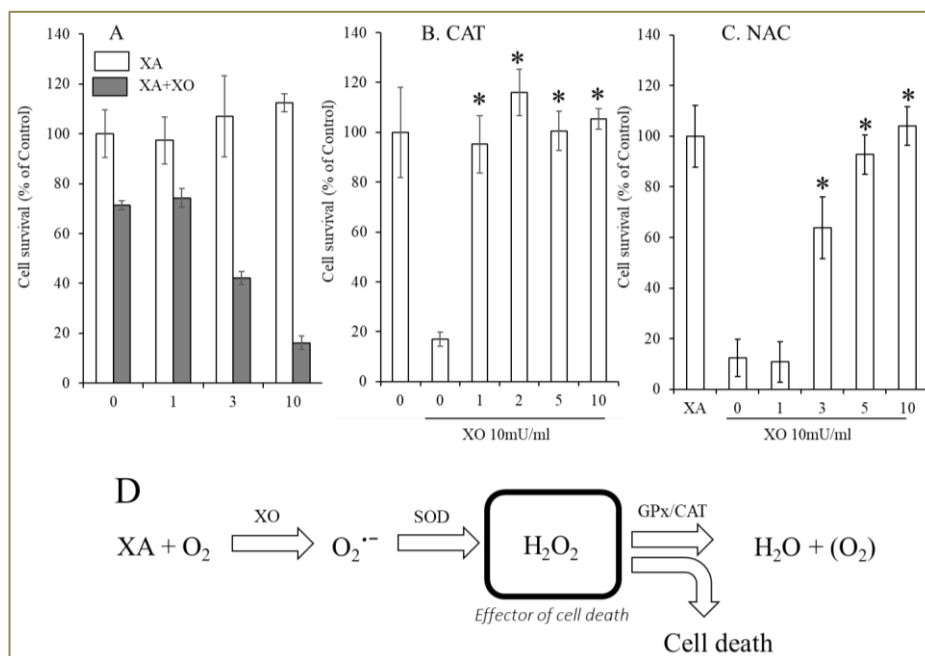


FIGURE 1. Cytotoxicity of XA/XO and the protective effects of CAT and NAC. (A) Toxic effects by XA/XO. Various concentrations (0, 1, 3, and 10 mU/ml) of XO were added to the medium without (white columns) or with (black columns) 1 mM XA, incubated for 24 h, and then measured for cell survival by performing the MTT assay as described in Materials and Methods. Data are the mean \pm SD ($n = 4$). Significance of differences between cells [XA (+) and XO (+)] and those [XA (+) and XO (−)] was determined by using Student's t test (*, $p < 0.05$). Survival of cells [XA (−) and XO (−)] was set as the control (100%). (B) Protective effects by CAT. Various concentrations of CAT were added 30 min before 10 mU/ml XO was added or not to the medium containing 1 mM XA. The cells were then incubated for 24 h, after which cellular survival was measured by use of the MTT assay. Data are the mean \pm SD ($n = 4$). Significance of differences relative to cells [XA (+) and XO (+)] was determined by using Student's t test (*, $p < 0.05$). Survival of cells [XA (+) and XO (−)] was set as the control (100%). (C) Protective effects by NAC. Various concentrations of CAT were added 30 min before 10 mU/ml XO was added to the medium containing 1 mM XA. The cells were then incubated for 24 h, and thereafter cellular survival was measured by using the MTT assay. Data are the mean \pm SD values ($n = 4$). Significance of differences relative to cells [XA (+) and XO (+)] was determined by using Student's t test (*, $p < 0.05$). Survival of cells in the control group [XA (+) and XO (−)] was set as 100%. (D) Mechanism of toxic effects by XA/XO. Superoxide (O₂^{•−}) is produced by XA/XO in the extracellular space and may enter the cells and be converted to H₂O₂ by SOD intracellularly. Extracellular superoxide may also undergo auto-dismutation to form H₂O₂ which then readily enters the cells. In addition, XA/XO can directly form H₂O₂ via 2-electron reduction of O₂ (not shown). When its level is below the capacity of the cellular defense system, H₂O₂ is destroyed mainly by GPx and CAT. However, when it is overproduced, H₂O₂ can initiate the cell death pathway.

2.2. COS7 Cell Culture and Cytotoxicity Assay

COS7 cells, a simian malignant tumor cell line, were cultured as described elsewhere [17, 18]. The cells were maintained in 10-cm dishes (Invitrogen) con-

taining 10 ml of Dulbecco's Modified Eagle medium supplemented with 10% (v/v) heat-inactivated (56°C, 30 min) fetal calf serum (Invitrogen). The cells were seeded into 24-well plates at a density of 4×10^4 cells/cm². When examining the protective effects on

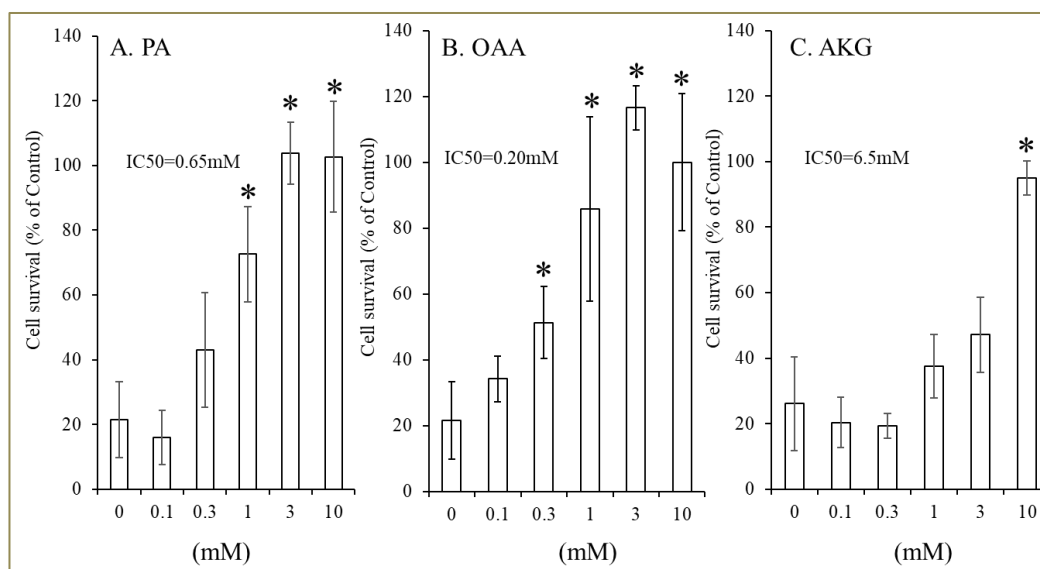


FIGURE 2. Protective effects of PA (A), OAA (B), and AKG (C) on XA/XO-induced cell death. Various concentrations (0, 0.1, 0.3, 1.0, 3.0, and 10.0 mM) of PA, OAA, and AKG were singly added 30 min before 10 mU/ml of XO was added or not to the medium containing 1 mM XA. Then the cells were incubated for 24 h, and cell survival was subsequently measured by performing the MTT assay as described in Materials and Methods. The calculated IC_{50} values were 0.65, 0.20, and 6.5 mM, respectively. Data are mean \pm SD ($n = 4$). Significance of differences relative to cells [XA (+) and XO (+)] was determined by using Student's *t* test (*, $p < 0.05$). Survival of control cells [XA (+) and XO (-)] was set at 100%.

XA/XO-induced toxicity, the medium was changed to that containing 1 mM XA after a 24-h incubation. Then various concentrations of CAT, NAC, or organic acids were added, and 30 min later, 10 mU/ml of XO was added. Thereafter, the cells were incubated for an additional 24 h. To evaluate survival of the cells, we performed the MTT assay according to the procedures described before [17, 18].

2.3. Dichlorofluorescein (DCF) Assay

The extent of cellular oxidative stress was assessed using the DCFH-DA assay, as described elsewhere [19, 20]. Cells were plated 24 h before initiation of the experiment at a density of 4×10^4 cells/well in 24-well plates. The media were changed to those containing a given organic acid, 10 μ M DCFH-DA, and 1 mM XA. The plate was immediately set into a Spark10M device (Tecan Japan, Tokyo, Japan) under an atmosphere of 5 % CO_2 and at 37°C. Next, 10 mU/ml of XO or vehicle was added to the wells at 30 min, and the cells were incubated further for 150 min.

DCF fluorescence was measured at a 485-nm excitation wavelength and 538-nm emission wavelength at 10-min intervals. Fluorescence values were expressed as a percentage of the value for the untreated control just containing 1 mM XA [19, 20].

2.4. Statistical Analysis

Experiments presented herein were repeated at least 3 times with each experiment performed in quadruplicate. Data were presented as the mean \pm standard deviation (SD). The statistical significance of differences was examined by performing Student's *t*-test, and considered at a *p* value $< 0.5\%$.

3. RESULTS

3.1. H_2O_2 as an Effector of Cell Death

Physiologically, superoxide is produced from aerobic metabolism of mitochondria, and it is removed by cellular defense systems. This is why we employed

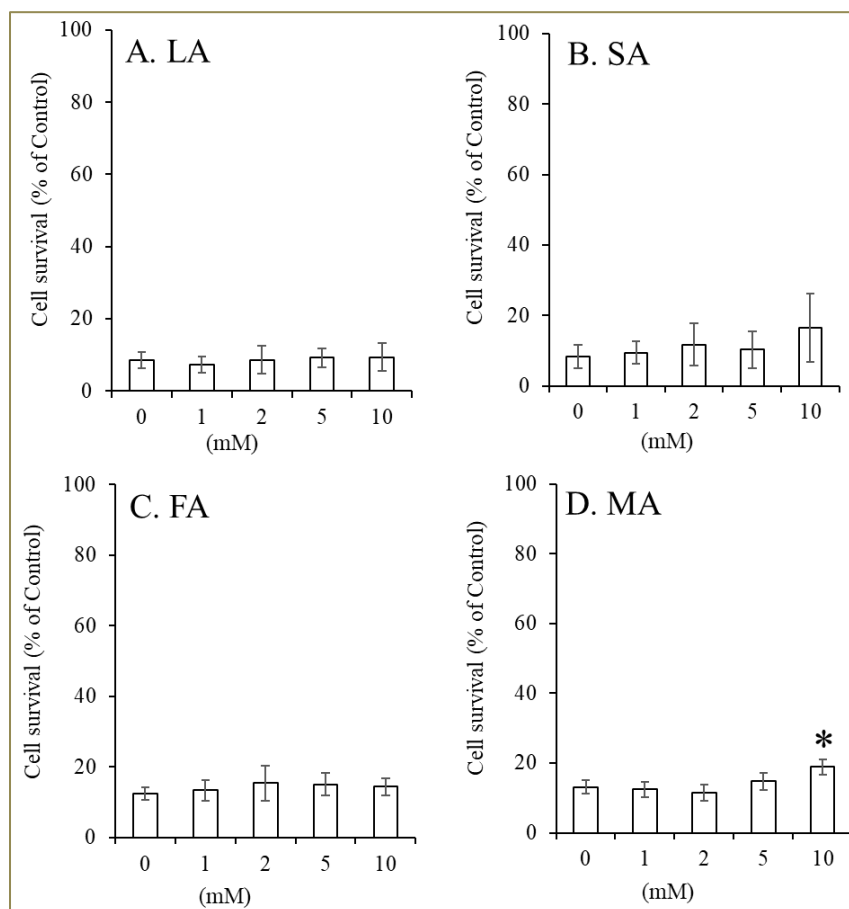


FIGURE 3. Effects of LA (A), SA (B), FA (C), and MA (D) on XA/XO-induced cell death. Various concentrations (0, 1.0, 2.0, 5.0, and 10.0 mM) of LA, SA, FA, and MA were added singly 30 min before 10 mU/ml XO was added or not to the medium containing 1 mM XA. After incubation of the cells for 24 h, cell survival was measured by use of the MTT assay as described in Materials and Methods. Data are the mean \pm SD ($n = 4$). Significance of differences relative to cells [XA (+) and XO (+)] was determined by using Student's t test (*, $p < 0.05$). Survival of control cells [XA (+) and XO (-)] was set at 100%.

XA/XO as an inducer of oxidative stress. First, the XA/XO-induced cell death was confirmed by the results of the MTT assay (**Figure 1A**). In the absence of XA, the addition of XO did not trigger the death of COS7 cells; whereas it induced significant cell death in the presence of XA. A concentration of 10 mU/ml of XO killed almost all of the cells, whereas XO at 3 mU/ml was only partially effective. Next, we examined the effects of NAC and CAT on the cell death (**Figure 1B** and **1C**), both of which have been reported to destroy H_2O_2 , in order to identify the effector of the cell death. Either CAT (1.0–10

mU/ml) or NAC (3.0–10 mM) reduced the extent of cell death. These results suggest that H_2O_2 was an effector of the XA/XO-induced cell death (**Figure 1D**).

3.2. Cytoprotective Effects of α -Keto Acids

Pretreatment of the cells for 30 min with α -keto acids at concentrations between 0.1 and 10.0 mM protected against cell death, but protective potencies varied with different α -keto acids (**Figure 2**). The potencies of protection (IC_{50} values) by α -keto acids PA, OAA,

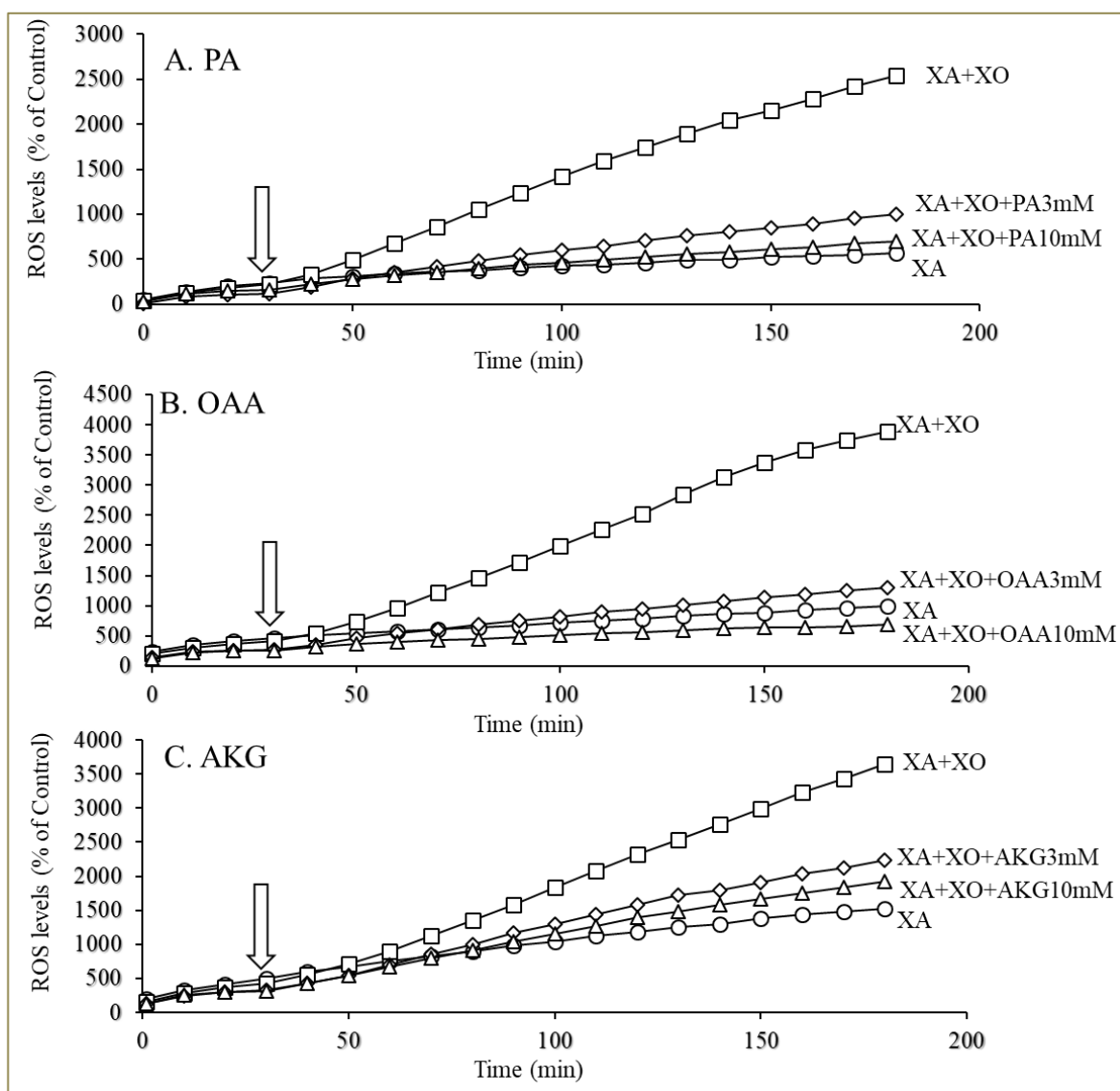


FIGURE 4. Antioxidant effects of PA (A), OAA (B), and AKG (C) against ROS burst induced by XA/XO. DCFH-DA and the desired organic acid (PA, OAA, or AKG) at 3 or 10 mM was added to the culture medium, and the plate was immediately set into the Spark10M device. XO (10 mU/ml) or vehicle (PBS) was added automatically to the medium with 1 mM XA at 30 min as shown by the arrows in the graphs. Data are the mean. Because the SD values were within the symbols, they were omitted.

and AKG were 0.65, 0.2, and 6.5 mM, respectively. Other organic acids tested (LA, SA, FA, and MA) did not protect the COS7 cells against the XA/XO-mediated toxicity (Figure 3), though these organic acids can produce PA, OAA, or AKG by dehydrogenase systems in the mitochondria.

3.3. Antioxidant Effects of α -Keto Acids

As the protective effects of the KCIs may be due to the α -keto acid chemical structure rendering direct interaction with H_2O_2 inside the cells [21–28], we next examined whether these organic acids could re-

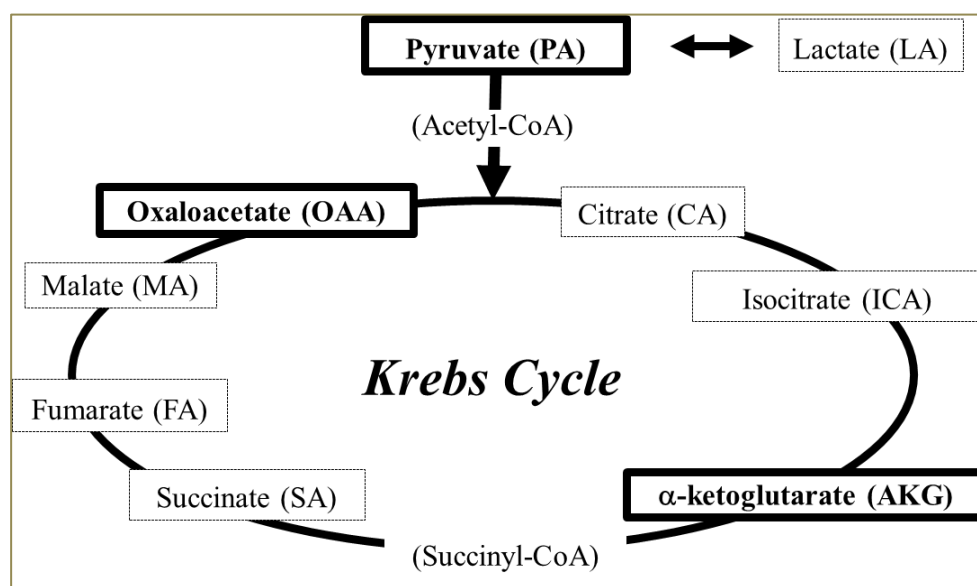


FIGURE 5. KCIs and the common chemical group of protective organic acids (PA, OAA, and AKG). PA, OAA, and AKG all have the common α -keto acid group, which can directly react with H_2O_2 .

duce the level of ROS in COS7 cells (Figure 4). We quantified the levels of ROS with an ROS-sensitive fluorescent indicator, DCFH-DA, by use of a Spark 10M device (Tecan Japan). Based on the fluorescence activity, XO at 10 mU/ml increased the level of ROS by 20–40-fold, with the level plateaued at 45–60 min of exposure. PA, OAA, and AKG each lowered intracellular ROS levels, suggesting that these KCIs used at millimolar concentrations effectively reduced the ROS level in the cells.

4. DISCUSSION

We found that the α -keto acid group-containing KCIs (PA, OAA, and AKG) could protect COS7 cells against the toxic effects of XA/XO, possibly through direct interaction with H_2O_2 . Because DCFH-DA is hydrolyzed by cytosolic esterases and then reacts with ROS in the cytoplasm, inhibition of the increase in ROS levels by PA, OAA, or AKG could be due to their H_2O_2 -scavenging effect inside the cells. The conclusion of this study is illustrated in Figure 5. These organic acids appear to directly react with H_2O_2 , and this chemical reaction may be important for various biological actions of the com-

pounds. A number of studies [21–28] have demonstrated that PA, OAA, and AKG act both as a source of energy and as antioxidant agents to improve physiological metabolism and to scavenge ROS, thereby alleviating oxidative stress via non-enzymatic oxidative decarboxylation during H_2O_2 decomposition. Indeed, PA [21, 22], OAA [23, 24], and AKG [25, 26] exhibit protective effects against H_2O_2 -induced toxicity in vivo and in vitro and can cross the blood-brain barrier to scavenge H_2O_2 , thus providing a novel therapeutic approach against H_2O_2 -induced brain pathologies [27, 28].

5. CONCLUSIONS

The present study led us to the following 2 conclusions. First, we found that H_2O_2 is an effector of the cell death induced by the XA/XO system, even though it produces both superoxide and H_2O_2 in the extracellular space. Secondly, we discovered that among the organic acids involved in aerobic energy metabolism, α -keto acids (PA, OAA, and AKG) could protect COS-7 cells against XA/XO-induced toxic effects by reducing the H_2O_2 level in the cells. These results suggest that activated aerobic energy

metabolism in mitochondria, which can produce high levels of α -ketoacids, is a powerful cellular defense system against oxidative stress.

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The authors declare no conflicts of interest.

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