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Cinnabarinic Acid Generated from 3-Hydroxyanthranilic Acid Strongly Induces Apoptosis in Thymocytes through the Generation of Reactive Oxygen Species and the Induction of Caspase

Rie Hiramatsu,[1] Toshiaki Hara,[1] Hidetoshi Akimoto,[2] Osamu Takikawa,[2] Tsutomu Kawabe,[1] Ken-ichi Isobe,[3] and Fumihiko Nagase[1]*

[1]Department of Medical Technology, Nagoya University School of Health Sciences, Nagoya, Aichi, Japan
[2]National Institute of Longevity Science, National Center for Geriatrics and Gerontology, Obu, Aichi, Japan
[3]Department of Immunology, Nagoya University Graduate School of Medicine, Nagoya, Aichi, Japan

Abstract 3-Hydroxyanthranilic acid (3HAA) is one of the tryptophan metabolites along the kynurenine pathway and induces apoptosis in T cells. We investigated the mechanism of 3HAA-induced apoptosis in mouse thymocytes. The optimal concentration of 3HAA for apoptosis induction was 300–500 μM. The induction of apoptosis by a suboptimal concentration (100 μM) of 3HAA was enhanced by superoxide dismutase (SOD) as well as $MnCl_2$ and further promoted in the presence of catalase. The 3HAA-mediated generation of intracellular reactive oxygen species (ROS) was enhanced by SOD or $MnCl_2$ and inhibited by catalase. Corresponding to apoptosis induction, the generation of cinnabarinic acid (CA) through the oxidation of 3HAA was enhanced by SOD or $MnCl_2$ in the presence of catalase. The synthesized CA possessed more than 10 times higher apoptosis-inducing activity than 3HAA. The intracellular ROS generation was induced by CA within 15 min and decreased to the control levels within 4 h, whereas the 3HAA-induced ROS generation increased gradually up to 4 h. Corresponding to ROS generation, the mitochondrial membrane potential was downregulated within 15 min and retained by the CA treatment. Apoptosis induction by 3HAA or CA was dependent on caspases, and caspase-3 was much more strongly activated by CA than 3HAA. In conclusion, the CA generated from 3HAA possesses a strong apoptosis-inducing activity in thymocytes through ROS generation, the loss of mitochondrial membrane potential, and caspase activation. J. Cell. Biochem. 103: 42–53, 2008.

Key words: cinnabarinic acid; 3-hydroxyanthranilic acid; apoptosis; tryptophan metabolite; thymocytes; reactive oxygen species; mitochondrial membrane potential; caspase

There is ample evidence to show that the tryptophan metabolism along the kynurenine pathway is significantly involved in T-cell regulation [Grohmann et al., 2003; Moffett and Namboodiri, 2003; Mellor and Munn, 2004]. Two possible mechanisms of T-cell regulation by the tryptophan metabolism are proposed. One is the depletion of tryptophan required for T-cell survival [Munn et al., 1999; Hwu et al., 2000] and the other is the growth inhibition or apoptosis induction by tryptophan metabolites [Okuda et al., 1998; Morita et al., 1999, 2001; Fallarino et al., 2002; Frumento et al., 2002; Terness et al., 2002]. Of the tryptophan metabolites, 3-hydroxyanthranilic acid (3HAA) is a potent inducer of apoptosis in T cells through mitochondrial cytochrome *c* release and the activation of caspase-8 [Fallarino et al., 2002]. The T-cell apoptosis that is induced by 3HAA is additively increased by other tryptophan metabolites,

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*Correspondence to: Fumihiko Nagase, Department of Medical Technology, Nagoya University School of Health Sciences, 1–20 Daikominami-1-chome, Higashi-ku, Nagoya, Aichi, 461-8673, Japan.
E-mail: nagase@met.nagoya-u.ac.jp

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such as kynurenine and 3-hydroxykynurenine, and further enhanced by the co-stimulation of TCR complex [Terness et al., 2002].

The detailed mechanism of the T-cell apoptosis that is caused by 3HAA has not yet been fully elucidated because of its unique redox ability. 3HAA can function as a reducing agent and a powerful peroxyl radical scavenger [Christen et al., 1992; Thomas and Stocker, 1999]. 3HAA is also known to be oxidized easily by O_2 to generate cinnabarinic acid (CA), a phenoxazinone derivative, as a major product [Ogawa et al., 1983a]. The mechanism of CA formation is proposed in Scheme 1, where the two-step oxidation of 3HAA by O_2 generates the quinone imine via anthranilyl radical and the imine is then condensed with secondary 3HAA to generate CA and H_2O_2 [Ogawa et al., 1983b; Dykens et al., 1987; Ishii et al., 1990; Manthey et al., 1990]. This Scheme is consistent with the fact that the CA production is enhanced by not only superoxide anion scavenger, superoxide dismutase (SOD) and manganese ions, but also by H_2O_2 scavengers and catalase [Ogawa et al., 1983a,b; Dykens et al., 1987; Ishii et al., 1990; Manthey et al., 1990; Christen et al., 1992; Liochev and Fridovich, 2001].

On the other hand, 3HAA has been shown to be toxic to bacteria and the toxicity is enhanced by SOD but inhibited by catalase, suggesting that the cytotoxicity is due to the H_2O_2 generated from the oxidation of 3HAA [Ishii et al., 1991]. However, it has been shown that CA itself is cytotoxic to bacteria [Eggert, 1997] and that actinomycin D, an analogue of CA, is a strong apoptosis inducer [Adrain et al., 2001; Caserta et al., 2003]. It is thus likely that CA may play an additional role in the 3HAA-induced apoptosis in thymocytes. With this in mind, we have reanalyzed the 3HAA-mediated apoptosis in thymocytes and found that the CA generated from 3HAA shows more than 10 times higher apoptosis-inducing activity than 3HAA. We herein show the mechanism of CA-induced apoptosis in thymocytes.

MATERIALS AND METHODS

Reagents

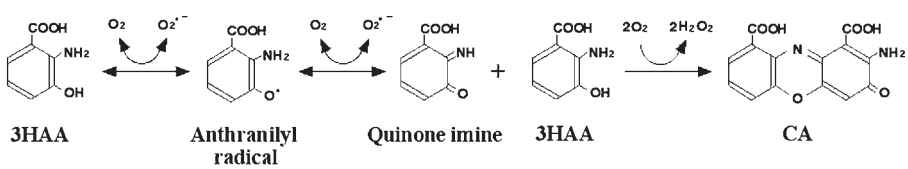
The 3HAA, N-acetyl-L-cystein (NAC), catalase, SOD and 3,3-dihexyloxacarbocyanine iodide (DiOC6(3)) were purchased from Sigma-Aldrich (St. Louis, MO). The Z-VAD-FMK (Z-VAD) was purchased from Calbiochem (Darmstadt, Germany). The hydroethidine (HE)^{TM} fluorescent stain was purchased from Polysciences, Inc. (Warrington, PA). The anti-cleaved caspase-3 (Asp 175) antibody was purchased from Cell Signaling Technology, Inc. (Beverly, MA).

Cell Cultures

The suspension of the thymocytes from the BALB/c mouse (Japan SLC, Shizuoka, Japan) was prepared for the in vitro cultures. The medium used for the cell culture (culture medium) is RPMI1640 medium supplemented with 10% FCS, 2.0 mM glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The thymocytes (10^6/well) were cultured in 0.2 ml of culture medium on 96-well plates for the assay of DNA fragmentation, reactive oxygen species (ROS) generation, and mitochondrial membrane potential by flow cytometry. For the assay of DNA fragmentation by electrophoresis, assay of CA and Western blot, cell cultures were expanded at the same concentration of cells.

Flow Cytometry (FCM)

DNA fragmentation was assayed as described previously [Du et al., 2000]. Stimulated cells were harvested and resuspended in propidium iodide (PI) buffer (0.1% Triton X-100, 0.1% trisodium citrate, and 50 μg/ml PI). After 15 min of incubation, the degree of DNA fragmentation was measured by an EPICS XL flow cytometer (Beckman Coulter, Inc., Fullerton, CA). The nuclei to the "left" of the G1 peak were considered apoptosis. The ROS generation was



Scheme 1. The oxidation of 3-hydroxyanthranilic acid to cinnabarinic acid.

assessed using HE as described previously [Du et al., 2000]. Oxidation of HE produces ethidium, which can emit red fluorescence after intercalation with cellular DNA. The stimulated cells were harvested and incubated with 2 μM HE for 15 min at 37°C. After washing with PBS twice, the cells were analyzed by flow cytometry selecting live cells by gating forward scatter. The loss of the mitochondrial membrane potential was assessed using DiOC6(3) [Castedo et al., 2002]. The stimulated cells were harvested and incubated with 40 nM DiOC6(3) for 15 min at 37°C. After washing with PBS twice, the cells were analyzed by flow cytometry selecting live cells by gating forward scatter or PI staining.

Electrophoresis

The cells were lysed in hypotonic lysing buffer (50 mM Tris-HCl, 0.5% sodium dodecyl sulfate (SDS), 10 mM EDTA) and incubated for 1 h at 55°C. Aliquots of 20 μl of samples were mixed with 6 μl of 0.25% (w/v) bromophenol blue and 40% (w/v) sucrose, and run on 2% (w/v) agarose gels with 0.1 μg/ml ethidium bromide, as described previously [Nagase et al., 1998].

Synthesis of CA

CA was prepared according to the method by [Prinz and Savage, 1977]. The 3HAA (100 mg) was dissolved in aqueous N, N′-dimethylformamide (3.2 ml dimethylformamide + 1 ml H_2O). To this solution were added, with stirring, $NaH_2PO_4 \cdot 2\ H_2O$ (0.48 g) and $Na_2HPO_4 \cdot 12\ H_2O$ (0.35 g), followed by "active" MnO_2 (0.3 g). The reaction mixture was stirred at room temperature for 90 min and then drowned in a solution of ferrous sulphate (0.6 g $FeSO_4 \cdot 7\ H_2O$) in 2N HCl (20 ml). The bright red precipitate of CA was then allowed to settle. Next, the product was removed by centrifugation, washed with water and dried. An analysis of the synthesized CA by HPLC showed more than 95% purity.

Assay of CA

The cells were cultured in the culture medium without phenol red (Sigma-Aldrich). The culture supernatants were collected and the UV-visible spectra were measured by a UV-vis-NIR recording spectrophotometer. Before the HPLC assay, the culture supernatants were acidified with trichloroacetic aicd (TCA) to 5% (w/v) final concentration (1:6, vol/vol). The mixture was centrifuged at 10,000 rpm for 10 min and then the supernatants were collected. The HPLC analyses were performed on a liquid chromatography system (LC-20 pump, SPD-M20A photodiode array detector; Shimazu, Kyoto, Japan). The reaction products were separated by an HPLC column (Intertsil ODS-3, 4.6 mm × 150 mm, 0.3 μm; GL Science, Tokyo, Japan) using a 60-min linear gradient of 0–100% methanol in 0.086% (v/v) phosphoric acid. The flow rate was 1 ml/min at 25°C and the elution profile was monitored at 450 nm.

Western Blotting

A Western blot analysis was carried out as described previously [Du et al., 2000]. In brief, the cells (10^7/100 μl) were lysed with an equal volume of 2× sample buffer and boiled for 3 min. The cell lysates were then passed though a syringe with a 26G needle before being applied on 10% SDS–polyacrylamide gels. Next, the protein-transferred membrane was incubated with the first antibody, followed by the anti-rabbit IgG antibody conjugated to horseradish peroxidase (Amersham Life Science, Boston, MA), which was visualized using a Western blot chemical reagent, Renaissance (NEN, Boston, MA).

RESULTS

Caspase-Dependent and Redox-Regulated Induction of Apoptosis in Thymocytes by the Stimulation With 3HAA

The induction of apoptosis in thymocytes by 3HAA was studied. The FCM assay showed that 300–500 μM 3HAA induced the highest DNA fragmentation in 18 h cultures (Fig. 1A). However, a higher concentration of 3HAA (1 mM) did not induce DNA fragmentation. Correspondingly, the electrophoresis assay showed the induction of strong DNA fragmentation by 300–500 μM but not by 1 mM 3HAA (Fig. 1B). The high (1 mM) and optimal (300 μM) concentrations of 3HAA induced 45.8 and 8.6% cell death, respectively, thus suggesting a switch from apoptosis to necrosis after stimulation with 1 mM 3HAA. These results show that the optimal concentrations of 3HAA for the induction of apoptosis are 300–500 μM. The effects of an antioxidant, NAC, and a caspase inhibiter, Z-VAD, on the induction of apoptosis by 3HAA were studied. NAC (20 mM) and Z-VAD (10 μM)

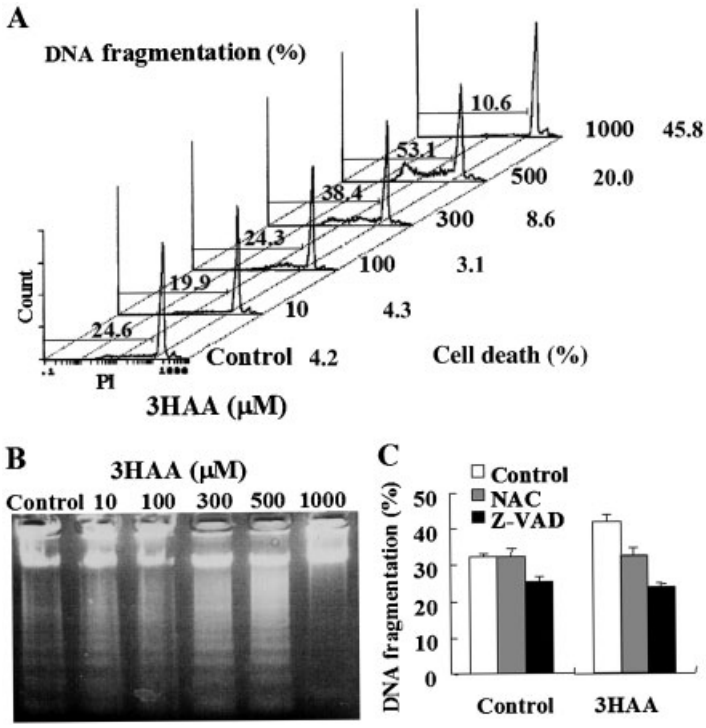


Fig. 1. Apoptosis induction in thymocytes stimulated with 3HAA and its prevention by NAC or Z-VAD. Thymocytes were incubated with or without (**A**,**B**) 10–1000 μM 3HAA or (**C**) 300 μM 3HAA together with or without 20 mM NAC or 10 μM Z-VAD for 18 h. DNA fragmentation of the thymocytes was assayed by (A,C) FCM or (B) electrophoresis. A: Percentage of cell death of the thymocytes was assayed by trypan blue exclusion test. C: The means +/− SD of the percentage of DNA fragmentation in the triplicate cultures are presented.

inhibited the induction of apoptosis by 300 μM 3HAA (Fig. 1C). These results indicate that the induction of apoptosis in thymocytes by 3HAA is therefore redox-regulated and caspase-dependent.

The Enhancement of 3HAA-Induced Apoptosis in Thymocytes by SOD or $MnCl_2$ and its Promotion by Catalase

SOD and catalase are antioxidative enzymes. On the other hand, $MnCl_2$ as well as SOD have the ability to enhance the oxidation of 3HAA [Ogawa et al., 1983b; Dykens et al., 1987]. Therefore, the effects of SOD, $MnCl_2$, or catalase on apoptosis induction by various concentrations of 3HAA were studied in thymocytes (Fig. 2). The SOD increased the induction of apoptosis by 62% with a suboptimal concentration (100 μM) of 3HAA, however, completely inhibited the induction of apoptosis by the optimal concentration (300 μM) of 3HAA (Fig. 2A). Other experiments showed that 100–300 μM 3HAA alone increased the induction of apoptosis, but decreased it in the presence of SOD in a concentration dependent manner (data not shown). The percentages of cell death of thymocytes induced with 300 μM 3HAA alone, together with catalase, SOD, and catalase + SOD were 13.8, 12.1, 32.2, and 46.1%, respectively, thus suggesting the induction of necrosis but not the rescue of the cells by SOD. $MnCl_2$ also increased the induction of apoptosis by 40 and 80% at 50 and 100 μM 3HAA, respectively, and inhibited 300 μM 3HAA-mediated apoptosis induction to a lower level than that of the 3HAA-unstimulated control (Fig. 2B). The catalase slightly augmented the induction of apoptosis by 100 μM 3HAA in the presence of SOD or $MnCl_2$, but reversed the SOD- or $MnCl_2$-mediated inhibition of apoptosis by 300 μM 3HAA (Fig. 2A,B). In contrast, the catalase alone hardly influenced the 3HAA-mediated apoptosis induction. These results indicate that both SOD and $MnCl_2$ enhance a suboptimal concentration of 3HAA-mediated apoptosis induction and inhibit an optimal concentration of 3HAA-mediated apoptosis induction in thymocytes.

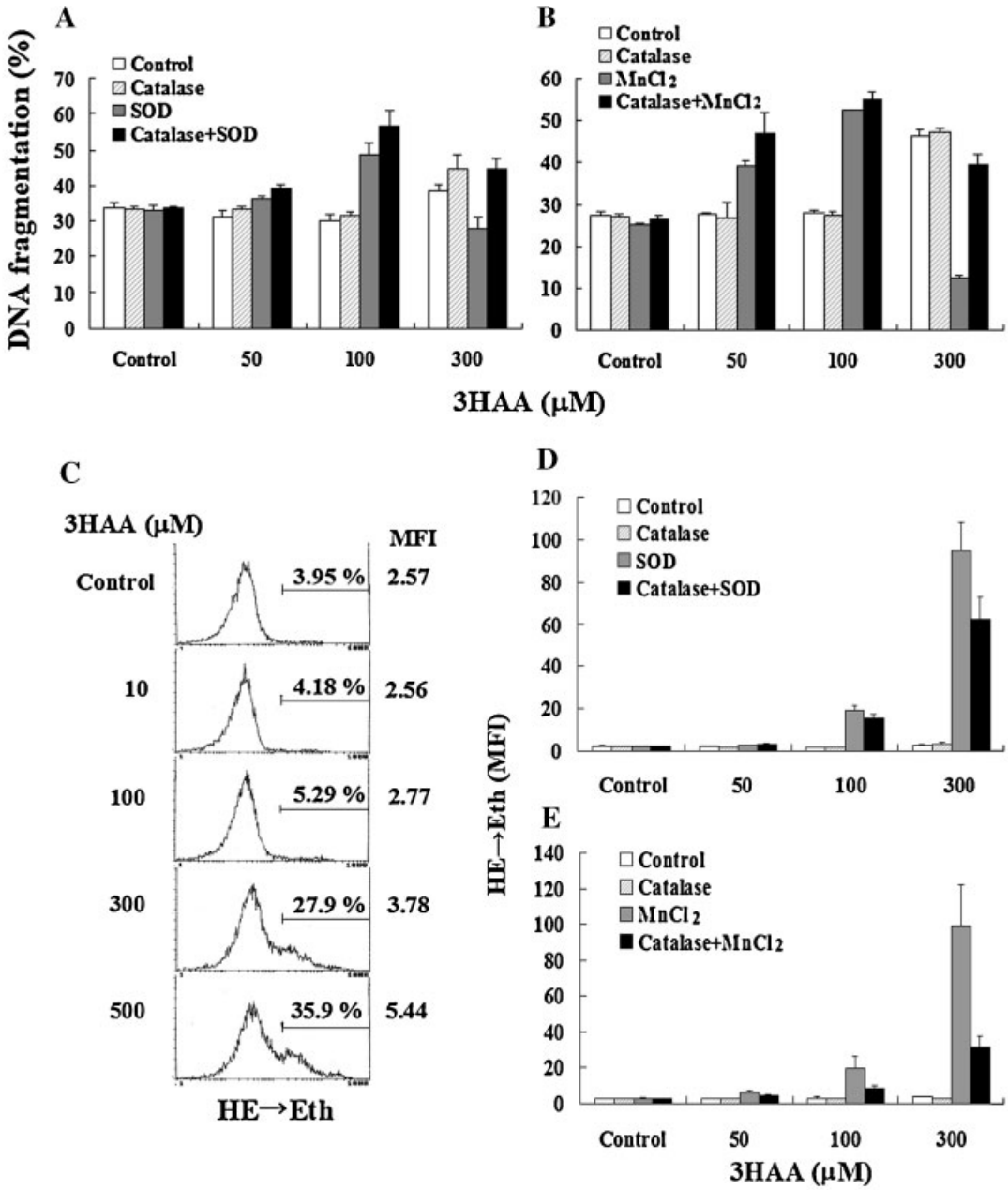


Fig. 2. The enhancement of 3HAA-induced apoptosis and intracellular ROS generation in thymocytes by SOD or $MnCl_2$. (**A**,**B**) The thymocytes were incubated with 50–300 μM 3HAA together with or without (A) 200 U/ml SOD or (B) 25 μM $MnCl_2$ in the presence or absence of 200 U/ml catalase for 18 h. **C**: The thymocytes were incubated with or without 10–500 μM 3HAA for 4 h. (**D**,**E**) The thymocytes were incubated with or without 50–300 μM 3HAA together with or without (D) 200 U/ml SOD or (E) 25 μM $MnCl_2$ in the presence or absence of 200 U/ml catalase for 4 h. (A,B) DNA fragmentation and (C,D,E) ROS generation were assayed by FCM. A: The percentage of cell death of thymocytes was assayed by trypan blue exclusion test. The means +/− SD of (A,B) the percentage of DNA fragmentation, (C,D,E) fluorescence intensity or (C) the percentage of the HE→Eth-positive cells in the triplicate cultures are presented.

The Enhancement of 3HAA-Mediated Intracellular ROS Generation in Thymocytes by SOD or $MnCl_2$ and its Prevention by Catalase

The SOD and $MnCl_2$ both augmented a suboptimal concentration of 3HAA-mediated apoptosis induction and this enhancement was not inhibited by catalase, indicating that the extracellular H_2O_2 generated from 3HAA is not involved in this process. Therefore, the intracellular ROS generation in the induction of apoptosis by 3HAA was examined by FCM using

HE (Fig. 2C–E). More than 300 μM of 3HAA alone induced ROS generation in the thymocytes in a concentration-dependent manner as shown in the percentage of HE → Eth-positive cells or the mean fluorescence intensity (MFI) (Fig. 2C). The SOD (Fig. 2D) or $MnCl_2$ (Fig. 2E) enhanced the ROS generation by 100–300 μM 3HAA. The ROS generation that was induced by 300 μM 3HAA was enhanced 44-fold by the SOD and 26-fold by the $MnCl_2$ (Fig. 2D,E). These results suggest that SOD reacts to 3HAA as a ROS generator rather than as a SOD. In contrast, the catalase inhibited the SOD- or $MnCl_2$-mediated enhancement of the ROS generation in the thymocytes that were stimulated with 100–300 μM 3HAA. The NAC also inhibited the 3HAA-mediated ROS generation in the thymocytes (data not shown). These data show that both catalase and NAC act as antioxidants in 3HAA-mediated apoptosis induction. However, the 3HAA-mediated apoptosis induction was enhanced by catalase and inhibited by NAC, thus indicating that the actions of the catalase and NAC in 3HAA-mediated apoptosis induction are completely opposite.

The Enhancement of CA Generation Through 3HAA Oxidation by SOD or $MnCl_2$ in the Presence of Catalase

It has been shown that the oxidation of 3HAA and the generation of CA from 3HAA are increased by SOD [Ogawa et al., 1983b; Dykens et al., 1987; Ishii et al., 1990; Liochev and Fridovich, 2001], $MnCl_2$ [Ogawa et al., 1983a,b; Dykens et al., 1987] or catalase [Ogawa et al., 1983a,b; Dykens et al., 1987; Ishii et al., 1990; Manthey et al., 1990; Christen et al., 1992]. It is possible that the SOD-, $MnCl_2$-, or catalase-mediated augmentation of 3HAA-induced apoptosis is associated with the increase of 3HAA oxidation and the formation of CA. Therefore, the generation of CA during 3HAA oxidation in the presence of SOD, $MnCl_2$, or catalase was studied by measuring the absorption spectrum of the culture supernatants of the thymocytes after 6 h of incubation (Fig. 3). 3HAA at 300 μM alone oxidized and generated the CA as indicated by the increase of the absorption at 450 nm (Fig. 3A). Around 30 μM of CA was generated after 6 h of culture with 300 μM 3HAA, which was determined using the absorbance coefficient of CA at 450 nm (17,000 $M^{-1} \cdot cm^{-1}$) [Rao and Vaidyanathan, 1966]. The NAC at 20 mM completely inhibited the generation of CA (Fig. 3A). The SOD as well as $MnCl_2$ increased the absorbance at 450 nm in the supernatants of the cell cultures with 100 μM 3HAA (Fig. 3B,C). The catalase alone did not significantly increase the CA generation. However, the catalase markedly increased the absorbance at 450 nm in the supernatant of the cell culture in the presence of SOD or $MnCl_2$. The generation of CA from 3HAA was confirmed by HPLC, in which CA was eluted with a retention time of 36 min. Consistent with the findings of a spectrophotometric analysis, CA was generated from 100 μM 3HAA by either SOD or $MnCl_2$ in the presence of catalase (Fig. 3D), thus indicating again that catalase promotes SOD- or $MnCl_2$-mediated CA generation. The SOD or $MnCl_2$ alone induced a slight degree of CA generation, but a main peak with an absorption at 450 nm was eluted with a retention time of 34 min, which was different from the retention time of CA. These results suggest that either SOD or $MnCl_2$ alone increases the generation of an intermediate on the pathway from 3HAA to CA.

The Synthesized CA Demonstrated an Apoptosis-Inducing Activity That was 10 Times Higher Than That for 3HAA

Our results suggest that the CA that is generated from 3HAA through oxidation increases the 3HAA-mediated induction of apoptosis. Therefore, the ability of the synthesized CA to induce apoptosis was tested in the thymocytes. CA showed the single absorption peak around 450 (Fig. 4A). CA at 30–50 μM concentrations induced the highest apoptosis (Fig. 4B), whereas 300–500 μM 3HAA possessed the highest apoptosis-inducing activities as shown in Figures 1A,B. The kinetics of apoptosis induction by 3HAA or CA was studied in the thymocytes (Fig. 4C). Apoptosis was induced in the thymocytes within 6 h after the stimulation with 30 μM CA or 300 μM 3HAA, in which the CA induced higher apoptosis than the 3HAA. Therefore, CA is more than 10 times more active than 3HAA in the induction of apoptosis.

Rapid and Transient Induction of Intracellular ROS Generation in Thymocytes Stimulated With CA

The ability of the CA to induce ROS generation in the thymocytes was further studied (Fig. 5). Surprisingly, the ROS generation was

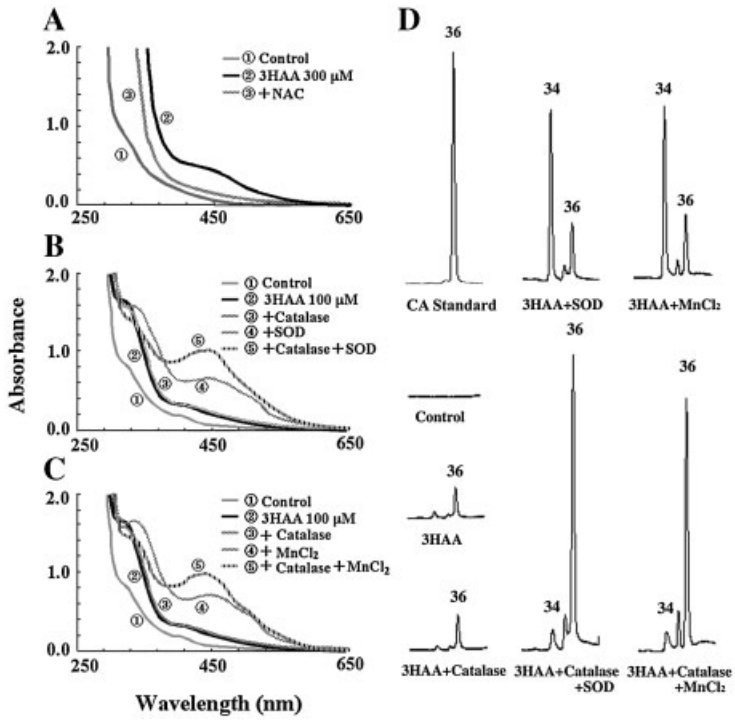


Fig. 3. The enhancement of CA generation though 3HAA oxidation by SOD or $MnCl_2$ and its promotion by catalase. **A**: The thymocytes were incubated with or without 300 μM 3HAA together with or without 20 mM NAC for 6 h. **B**,**C**,**D**: The thymocytes were incubated with or without 100 μM 3HAA together with or without (B,D) 200 U/ml SOD or (C,D) 25 μM $MnCl_2$ in the presence or absence of 200 U/ml catalase for 6 h. (A,B,C) The absorption spectra of the culture supernatants were recorded with a UV/VIS spectrophotometer and (D) the HPLC elution profiles of the supernatants were monitored by absorbance at 450 nm with retention time (min).

induced within 15 min in the thymocytes stimulated with 30 μM CA and decreased to the control levels within 4 h, whereas the ROS generation by 300 μM 3HAA was gradually increased in a time-dependent manner for 4 h (Fig. 5A). The rapid induction of ROS generation by CA was inhibited by NAC but not by SOD, catalase or their mixtures (Fig. 5B). Consistent with this was the fact that the CA-mediated induction of apoptosis was inhibited by NAC (Fig. 5C) but not SOD, catalase, or their mixtures (Fig. 5D). These results demonstrated the clear difference among the oxidative stress-inducing activities between CA and 3HAA. CA might induce ROS generation in thymocytes through a cellular mechanism which is different from the autoxidation of 3HAA.

Rapid Down-Regulation of Mitochondrial Membrane Potential in Thymocytes Stimulated With CA and its Retention

The loss of mitochondrial membrane potential and cytochrome *c* release from mitochondria are induced by ROS generation. The 3HAA induced the cytochrome *c* release from the mitochondria [Fallarino et al., 2002]. Therefore, the effects of CA on the mitochondrial membrane potential were tested. The mitochondrial membrane potential was disrupted 40% within 15 min and retained at least for 4 h in the thymocytes stimulated with 30 μM CA (Fig. 6A). However, 300 μM of 3HAA induced a weak disruption of the mitochondrial membrane potential. These results suggest that CA induces apoptosis in thymocytes through the rapid disruption of the mitochondrial membrane potential, corresponding to ROS generation.

High Levels of Caspase-3 Activation in Thymocytes Stimulated With CA

We studied whether or not CA induced apoptosis through caspase activation. The CA-induced apoptosis was inhibited by Z-VAD (Fig. 6B). Thirty micromolar of CA strongly induced both protein synthesis and the activation of caspase-3 in the thymocytes within 6 h

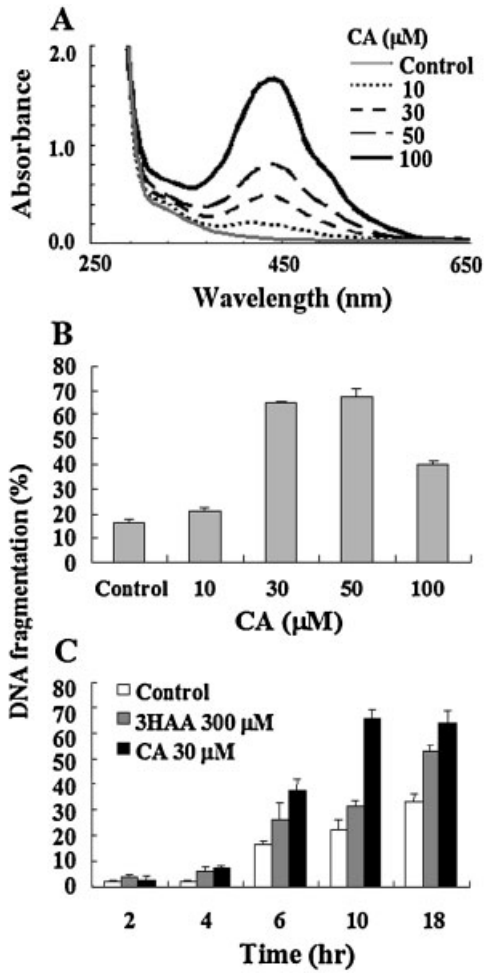


Fig. 4. The synthesized CA demonstrated an apoptosis-inducing activity that was 10 times higher than that for 3HAA. **A**: The absorption spectra of 10–100 μM of the synthesized CA in PBS were analyzed. **B**,**C**: The thymocytes were incubated with or without (B) 10–100 μM CA for 18 h, or (C) 300 μM 3HAA or 30 μM CA for 2–18 h. DNA fragmentation of the thymocytes was assayed (B) at 18 h or (C) at the indicated time of cultures by FCM. The means +/− SD of the percentage of DNA fragmentation in the triplicate cultures are presented.

(Fig. 6C). In contrast, 300 μM of 3HAA did not induce protein synthesis of caspase-3 and only weakly activated caspase-3. These results suggest that the strong apoptosis-inducing activity of CA is mediated through high levels of caspase-3 activation.

DISCUSSION

The present study showed that the 3HAA-mediated apoptosis of the thymocytes was enhanced by SOD or $MnCl_2$ alone and further promoted by catalase in the presence of SOD or $MnCl_2$. In this apoptosis, the SOD did not work as antioxidants and rather promoted the oxidation of 3HAA. There are two possible roles of SOD in the promotion. One is to promote the oxidation of 3HAA through the depletion of $O_2^{\cdot -}$ that is produced during the oxidation of 3HAA and inhibits CA generation by the backward reaction to 3HAA or the decomposition of CA by $O_2^{\cdot -}$ (Scheme 1), as reported previously [Dykens et al., 1987; Ishii et al., 1990; Manthey et al., 1990]. Another role of SOD in the promotion of 3HAA oxidation is a direct oxidation of 3HAA by SOD itself as shown by Liochev and Fridovich [2001]. They proposed that the Cu(II) of SOD is reduced by 3HAA to the Cu(I) form and then reoxidized by the $O_2^{\cdot -}$ generated by autoxidation of anthranilyl or other radicals on the pathway to the CA. The mechanism of $O_2^{\cdot -}$ depletion by SOD is involved in both of the models, whereas the mechanism of $O_2^{\cdot -}$ generation by SOD through the oxidation of the radicals is involved in only the latter model. Our results showed that the intracellular ROS was accumulated by the stimulation with 3HAA in the presence of SOD (Fig. 2). Considering that the reagent used for the detection of ROS reacts mainly with $O_2^{\cdot -}$ (Rothe and Valet, 1990), it is likely that the activity of SOD to deplete $O_2^{\cdot -}$ is lower than that of SOD to generate $O_2^{\cdot -}$. We thus conclude that the latter mechanism, namely, the direct oxidation of 3HAA by SOD, thus plays a role in the enhancement of apoptosis by SOD. The role of $MnCl_2$ in the enhancement of apoptosis of 3HAA appears to be essentially the same as that of SOD, that is, a direct oxidation of 3HAA by the Mn(III) generated through the oxidation of Mn(II) by $O_2^{\cdot -}$ [Ogawa et al., 1983b]. This conclusion is consistent with our observations that both the SOD and $MnCl_2$ promoted the induction of the 3HAA oxidation in a similar fashion (Fig. 2D,E).

It has been proposed that catalase promotes the oxidation of 3HAA and/or prevents the H_2O_2-mediated decomposition of CA through the depletion of the H_2O_2 that is produced during the oxidation of 3HAA [Ogawa et al., 1983b; Ishii et al., 1990]. On the other hand, it has been shown that 3HAA is oxidized to CA by the peroxidase activity of the catalase in the presence of H_2O_2 [Christen et al., 1992]. 3HAA oxidation was also enhanced by the catalase in the presence of the H_2O_2-generators, such as SOD or $MnCl_2$. Taken together, it is suggested that the oxidation of 3HAA is accelerated by the

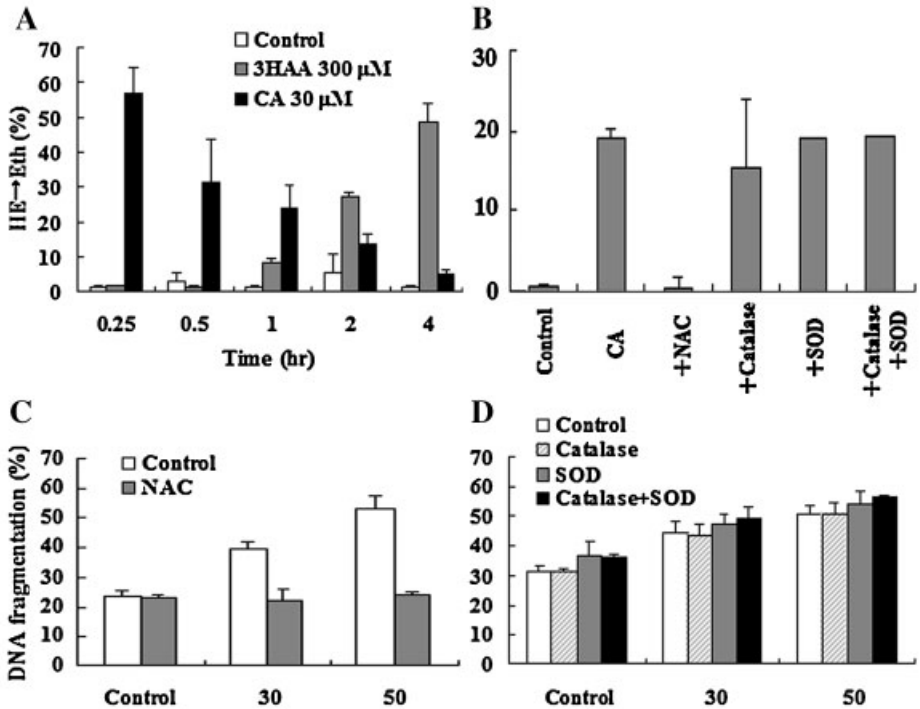


Fig. 5. Rapid and transient induction of intracellular ROS generation in the thymocytes stimulated with CA. **A**,**B**: The thymocytes were incubated with or without (A) 300 μM 3HAA or 30 μM CA for 15 min to 4 h or (B) 30 μM CA together with or without 20 mM NAC, 200 U/ml SOD, 200 U/ml catalase, or mixtures of catalase and SOD for 30 min. ROS generation in the thymocytes was induced at (A) the indicated time or (B) 30 min was assayed by FCM. **C**,**D**: The thymocytes were incubated with or without 30–50 μM CA together with or without (C) 20 mM NAC, or (D) 200 U/ml SOD, 200 U/ml catalase, or mixtures of catalase and SOD for 18 h. DNA fragmentation was assayed by FCM. The means +/− SD of (A,B) the percentage of HE → Eth-positive cells or (C,D) percentage of DNA fragmentation in the triplicate cultures are presented.

catalase through the depletion of H_2O_2 or peroxidase activity in the presence of SOD or $MnCl_2$.

We therefore conclude that CA generation is essential for the apoptosis induction in thymocytes by 3HAA on the basis of the following observations. First, an apoptosis-inducing concentration (300 μM) but not a suboptimal concentration (100 μM) of 3HAA alone significantly induced CA generation. Second, both the CA generation and apoptosis induction by 100 μM 3HAA in the thymocytes were enhanced by SOD or $MnCl_2$ in the presence of the catalase. Third, the CA possessed more than 10 times higher activity to induce apoptosis than 3HAA. Finally, the NAC inhibited the 3HAA-mediated apoptosis induction through the inhibition of CA generation. The apoptosis-inducing activity of CA was observed in Jurkat leukemia T cells (unpublished data), but not in human monocytic THP-1 cells [Morita et al., 1999]. These discrepancies may be explained by the differences of cell types or concentrations of CA used. As far as we know, this is the first report which identifies CA is a potent apoptosis inducer for both thymocytes and T cells.

We showed that the induction of apoptosis in the thymocytes by the optimal concentration (300 μM) of 3HAA was inhibited by the SOD or $MnCl_2$, corresponding to the extensive increase in ROS generation, and that these actions of the SOD and $MnCl_2$ were then reversed by the catalase. This activity of 3HAA may be related to the previous report that the SOD-mediated increase of 3HAA toxicity to bacteria is inhibited by catalase [Ishii et al., 1991]. Mn(II) as well as SOD enhances the oxidative damage of DNA by 3HAA through H_2O_2 generation [Hiraku et al., 1995]. Based on our findings, we speculated that the H_2O_2 was generated extensively during the oxidation of the high concentration of 3HAA, and switched from apoptosis to necrosis induction. However, the SOD or $MnCl_2$ alone did not generate CA as shown by the HPLC assay. Instead, a new intermediate was accumulated in the presence of the SOD or $MgCl_2$. We are presently trying to identify this new product in our laboratory.

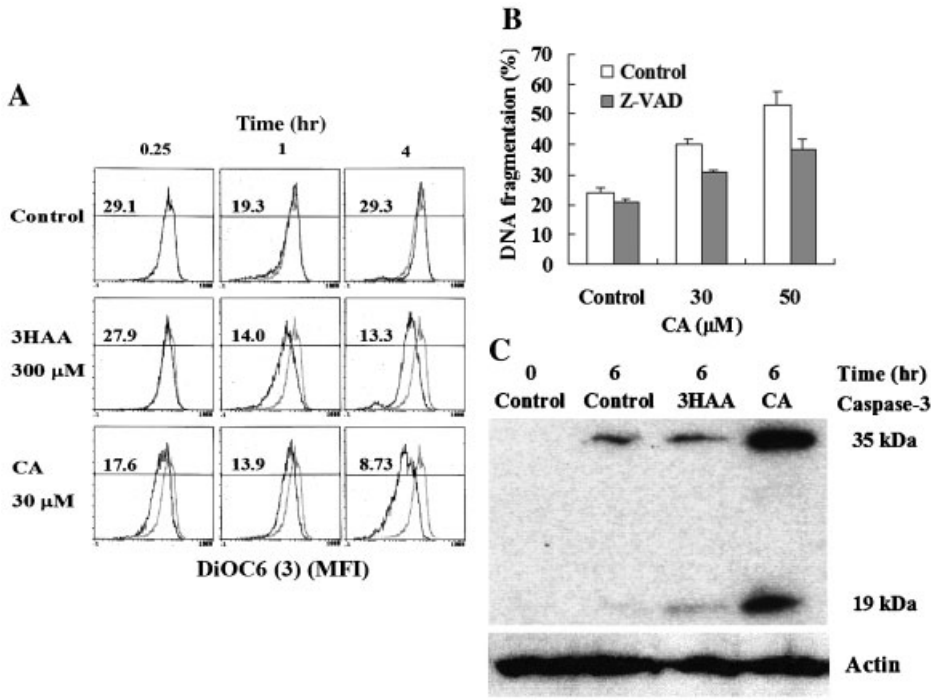


Fig. 6. Rapid downregulation of mitochondrial membrane potential and caspase-3 activation in thymocytes stimulated with CA. **A**: The thymocytes were incubated with or without 300 μM 3HAA or 30 μM CA for 15 min to 4 h. The mitochondrial membrane potential was analyzed by FCM with DiOC6(3) at the indicated time. **B**: The thymocytes were incubated with or without 30 μM CA together with or without 10 μM Z-VAD for 18 h. DNA fragmentation was assayed by FCM. The means +/− SD of the percentage of DNA fragmentation in the triplicate cultures are presented. **C**: The thymocytes were incubated with or without 300 μM 3HAA or 30 μM CA for 6 h. The caspase-3 activation was assayed by Western blot with anti-cleaved caspase-3 antibody detecting full-length caspase-3 protein (35 kDa) and cleaved caspase-3 protein (17, 19 kDa).

We showed that the ROS generation in the thymocytes was induced by CA within 15 min and downregulated to the control levels within 4 h, whereas the ROS generation induced by the 3HAA gradually increased during the 4-hr cultures. However, the levels of ROS generation in the thymocytes by 30 μM CA as well as 300 μM 3HAA were only a few percent of those induced by 300 μM 3HAA in the presence of SOD or $MnCl_2$, as shown in Figures 2D,E. Therefore, the rapid ROS generation rather than the levels of ROS generation may be characteristic of apoptosis induction by CA. The different patterns of kinetics of ROS generation suggested the different mechanisms of ROS generation by CA and 3HAA. In fact, the SOD, $MnCl_2$, or catalase did not significantly influence the CA-mediated apoptosis induction and ROS generation. These results suggest that the ROS generation and its downregulation induced in thymocytes by CA is well controlled by cellular mechanisms, whereas the ROS generation induced by 3HHA was found to correlate with 3HAA oxidation.

Corresponding to ROS generation, the mitochondrial membrane potential was rapidly downregulated in the thymocytes by CA and retained at least for 4 h. These results suggest that the loss of mitochondrial membrane potential and the rapid ROS generation induced by CA are linked for the induction of apoptosis in thymocytes.

The present study suggests that both types of inductions of apoptosis, by 3HAA and CA, are caspase-3-dependent. However, the ability of CA to activate caspase-3 was markedly higher than that of 3HAA. These results suggest that CA induces apoptosis in thymocytes through the rapid ROS generation and downregulation of the mitochondrial membrane potential following the strong activation of caspase-3. It has been shown that 3HAA induces apoptosis in mouse thymocytes through mitochondrial cytochrome *c* release and caspase-8 but not caspase-9 activation [Fallarino et al., 2002]. Further studies will be required in order to clarify the apoptosis-inducing mechanisms by CA and 3HAA. The actinomycin D-induced apoptosis

in Jurkat T cells results in cytochrome *c* release and the activation of caspase-3 [Adrain et al., 2001; Caserta et al., 2003] and is inhibited by Z-VAD and the caspase-3-specific inhibitor [Brown et al., 1999; Adrain et al., 2001; Caserta et al., 2003]. Therefore, CA and actinomycin D may induce apoptosis through a similar mechanism.

The evidence that the apoptosis-inducing activity of 3HAA is strongly enhanced by the generation of CA through oxidation is important for the regulation of T-cell responses by tryptophan metabolites along the kynurenine pathway. The excretion of 3HAA was induced by interferon-γ in human macrophages [Werner-Felmayer et al., 1989]. A high concentration of 3HAA accumulated in the local tissue environment of inflammation may be partially converted to CA by various enzymes or metal ions and acquire strong apoptosis-inducing activity. The IDO-induced tryptophan metabolites, such as kynurenine, 3-hydroxykynurenine, and 3HAA, additively inhibit T-cell proliferation through apoptosis induction [Terness et al., 2002]. The present study demonstrates that 3HAA acquires strong apoptosis-inducing activity by converting to CA through oxidation.

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