Indole-3-carbinol Generates Reactive Oxygen Species and Induces Apoptosis

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Cruciferous vegetables contain glucobrassicin which, during metabolism, yields indole-3-carbinol (I3C). The aim of this study was to find whether indole-3-carbinol caused apoptosis and its mechanism in Candida albicans. We found that treatment of Candida albicans with indole-3-carbinol significantly increased the reactive oxygen species and hydroxyl radical accumulation. The hydroxyl radical is one of the most active components of oxygen, and it is the end product of an oxidative damage cellular death pathway. We investigated the general phenotypes of apoptosis and then investigated whether there were other distinct markers of apoptosis. Furthermore, the effects of thiourea as a hydroxyl radical scavenger and protective effect of trehalose, which is the result of the fungal immune system, was also assured. This study indicates that indole-3-carbinol has apoptosis effects, including a production of hydroxyl radicals, cytochrome c release and activation of metacaspase. Both hydroxyl radicals and metacaspases triggered apoptosis in Candida albicans.

Key words  indole-3-carbinol; Candida albicans; apoptosis; hydroxyl radical; cytochrome c

The patients, who are immunocompromised due to cancer chemotherapy, organ or bone marrow transplantation, or human immunodeficiency virus infection, are at high risk of opportunistic infections.1) Among the opportunistic pathogens, the fungal species Candida albicans (C. albicans) is perhaps the most successful in humans. A commensal fungus that frequently colonizes on human mucosal surfaces, C. albicans has long been adapted to the human host and has evolved owing to the specific demands of the host environment. Distinctively, under conditions of immune system dysfunction, colonizing C. albicans strains can become opportunistic pathogens causing recurrent mucosal infections and life-threatening communicable infections with high mortality rates.2) The increasing emergence of strains of C. albicans resistant to the commonly used antifungal agents has made clinical management of candidiasis difficult. This has not only made the treatment of infectious diseases more problematic, but has also resulted in the reappearance of many diseases which were thought to be under control. To treat infections caused by multi-drug resistant bacteria and fungi, the improved drug therapies have become inevitable and urgent. Plants produce structurally diverse secondary metabolites, and it is thought to be a significant part of this chemical diversity serves to protect plants against microbial pathogens.3) In that respect, secondary metabolites of plant origin are important resources in developing new antimicrobial agents. Indole-3-carbinol (I3C), which was used in this research, is also one of these phytochemicals kinds of secondary metabolites.

I3C is found in all members of the cruciferous vegetable family, which includes cabbage, broccoli, Brussels sprouts, cauliflower and kale. Due to its anticarcinogenic effects in experimental animals and humans, I3C has received special interest as a potential chemopreventive agent. It has been found to inhibit the growth of various cancer cells, including those of the breast, prostate and colon.4—6) Most of these effects appear to occur because I3C modulates several nuclear transcription factors. Moreover, it has been elucidated that I3C has antimicrobial activity. In previous reports, we have shown the antimicrobial effects of I3C, including anti-bacterial and anti-fungal.7) Though I3C is known for its various biological activities, its antifungal effect and mode of action still needs to be investigated. In this study, we investigated whether I3C could exert antifungal effects through another mechanism like apoptosis process against Candida albicans.

MATERIALS AND METHODS

Reagents and Culture Conditions Indole-3-carbinol (I3C), Amphotericin B (AMB) and hydrogen peroxide (H2O2) used in this study, were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Stock solutions of I3C were prepared in dimethyl sulfoxide (DMSO) and stored at –20 °C. For all the experiments, a final concentration of 1% DMSO was used as the solvent carrier. Candida albicans (ATCC 90028) cells were cultured in a YPD broth (Difco) containing yeast extract, peptone, and dextrose (50 g/l) with aeration at 28 °C. The values represented the average of the measurements conducted in triplicate of three independent assays.

In Vitro Antimicrobial Activity Fungal cells (2×10⁶/ml) were inoculated into a YPD broth and dispensed into 0.1 ml/well in 96-well microtiter plates. Minimum inhibitory concentration (MIC) was determined by a serial two-fold dilution of test compounds, following a micro-dilution method. After 24 h of incubation at 28 °C, the minimal compound concentration that prevented the growth of a given test organism was determined and was defined as the MIC.5)

Intracellular Reactive Oxygen Species (ROS) Accumulation Intracellular ROS production and hydroxyl radical accumulation were measured using a fluorescent dye Dihydrorhodamine (DHR-123). Cells (2×10⁶/ml) were treated with 70 μM I3C, 9 μM AMB and 3 mM H2O2 for 2 h at 28 °C, based on the MIC value as a criterion. After incubation, the cells were washed with a phosphate buffered saline (PBS) before staining with 5 μg/ml of DHR-123 and analyzed by a
FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.).

**Annexin V Staining** Protoplasts of *C. albicans* were stained with fluorescein isothiocyanate (FITC)-labeled Annexin-V and propidium iodide (PI) by using the FITC-Annexin-V apoptosis detection kit. Cells (2 × 10^6/ml) were digested for 1 h at 28 °C in a potassium phosphate buffer (PPB) (pH 6.0) containing 20 mg/ml of lysing enzyme and 1 M sorbitol. Protoplasts were incubated with 70 mM I3C, 9 μM AMB, and 3 mM H_2O_2 for 1 h at 28 °C and incubated for 20 min in an Annexin binding buffer containing 5 μl of FITC-Annexin-V/ml and PI. Protoplasts were then examined by fluorescence microscopy, Axio Imager A1 and Axio Cam MR5 (Carl Zeiss). For the hydroxyl radicals quenching experiments, 150 mM of thiourea was added simultaneously with I3C. Thiourea has been used at millimolar levels in *C. albicans* as hydroxyl radical scavengers. They have shown that the level of intracellular trehalose is increased by I3C in a previous study. To find out about the protective effects of trehalose, it was added to the YPD media at a final concentration of 100 mM. *C. albicans* cells were incubated in YPD containing trehalose before they are used, since trehalose can be loaded enough. This concentration was referred to in previous literature. Thiourea and trehalose was used in all the subsequent tests.

**Measurement of DNA Damages** DNA strand breaks in *C. albicans* cells were analyzed by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method. Cells (2 × 10^6/ml) treated for 3 h with 70 μM I3C, 9 μM AMB, and 3 mM H_2O_2 were washed in a PBS, permeabilized for 2 min on ice, and washed again with a PBS. DNA ends were labeled with an 100 μM of I3C, 9 μM, and 3 mM H_2O_2 for 2 h at 28 °C. Subsequently, the cells were washed with a PBS, and incubated with 2 ng of DiOC6(3) for 30 min. Cells were analyzed by a flow cytometer.

**Measurement of Mitochondrial Membrane Potential (ΔΨ_m)** Fungal mitochondrial membrane depolarization was analyzed by 3,3′-dihexyloxacarbocyanine iodide [DiOC6(3)] staining. Cells (2 × 10^6/ml) were harvested and incubated with 70 μM I3C, 9 μM AMB, and 3 mM H_2O_2 for 2 h at 28 °C. Subsequently, the cells were washed with a PBS, and incubated with 2 μM of DiOC6(3) for 30 min. Cells were analyzed by a flow cytometer.

**Measurement of Metacaspase Activation** Metacaspases in *C. albicans* were measured using the CaspACE™ FITC-VAD-FMK In Situ Marker (Promega). Briefly, each substance treated cells were washed in a PBS, suspended in 200 μl staining solution containing 10 μM of CaspACE™ FITC-VAD-FMK In Situ Marker and incubated for 30 min at room temperature in the dark. Cells were then washed once and suspended in a PBS. Sample analysis was performed in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.) and fluorescence microscopy.

RESULTS AND DISCUSSION

**In Vitro Antimicrobial Activity** The antifungal effect of I3C on *C. albicans* was investigated and described in terms of the MIC. I3C showed antifungal activity against *C. albicans*. This compound exhibited MIC value of 70 μM, and the MIC value of this compound was as efficient as that of amphotericin B (AMB) or hydrogen peroxide (H_2O_2), showing MIC value of 9 μM or 3 mM on *C. albicans* (data not shown). We used AMB and H_2O_2 as a positive control to determine programmed cell death.

**Intracellular Reactive Oxygen Species (ROS) Accumulation** In the previous study, I3C had shown significant antifungal activity at low concentration conditions, which was similar to the level of AMB. The results had suggested that its antifungal effects were mediated by a cellular function, which does not need energy consumption and I3C had an effect on DNA. This means that there are many possible mechanisms of antifungal effects, including apoptosis nonphysiological effects. Hence, we have investigated that I3C could induce apoptosis in *C. albicans*. We determined the most efficient concentration of H_2O_2 to apoptosis. Apoptosis was accepted as a process not exclusive of multicellular but rather as a universal mechanism of cell elimination operating according to a basic program, including the simpler and more ancient forms of single-celled eukaryotes. The full apoptotic program comprises two phases and one of them has necrotic features. Therefore, we analyzed more definite sign of apoptosis process.
ROS are continuously formed as a consequence of cellular oxygen metabolism. Recent studies have suggested that the accumulation of ROS induce and regulate the induction of apoptosis in yeast. To determine the production and accumulation of intracellular ROS induce by I3C, we chose to utilize the ROS sensitive dye DHR-123, which has been used previously as a general indicator of cellular ROS levels. Multiple ROS directly oxidize DHR-123 into a highly stable, fluorescent derivative rhodamine-123 in such a way that an increase in the fluorescent signal reflects the ROS production. Cells treated with I3C exhibited high ROS levels compared to untreated cells. In the positive control assay, there was a significant increase of fluorescence when the cells were treated with AMB and H2O2 (Fig. 1). These results indicate that ROS induced by I3C accumulated in the interior of C. albicans cells.

**Annexin V Staining** Phosphatidylserine is only distributed in the inner leaflet of the lipid bilayer of the plasma membrane, which is maintained by the ATP-binding cassette transporters in *C. albicans*. The phosphatidylserine externalization at the outer leaflet of the cytoplasmic membrane is interpreted as an early marker of apoptosis in *C. albicans* cells as well as mammalian cells. To detect the early event of apoptosis and discrete necrosis, *C. albicans* cells exposed to I3C were co-stained with annexin V-FITC and PI. Cells exposed I3C were stained fluorescent green as annexin V-positive, displaying similar response to AMB and H2O2, an inducer of apoptosis in yeast cells (Fig. 2). However, cells treated with each substance for 1 h were rarely observed PI-positive (data not shown). To investigate the effects of thiourea and trehalose on apoptotic cell death, we added I3C with these oxidative stress protecting substances. This experiment was conducted based on the ideas of what hydroxyl radical, one of reactive oxygen species, contributed to cell death. Consequently, both of two showed phosphatidylserine is consistently situated inner membrane as expected. These results suggest that I3C induced apoptosis in *C. albicans*, as evidenced by a significant staining in annexin-V-positive apoptotic cells.

**Measurement of DNA Damages** To further confirm the apoptotic features induced in I3C treated *C. albicans*, the TUNEL assay was conducted in order to detect apoptotic DNA fragmentation by labeling 3’-OH termini with modified nucleotides catalyzed by terminal deoxynucleotidyl transferase. The labeling of DNA breaks by TUNEL, one of reliable methods for the identification of apoptotic cells, is uti-
lized to visualize the apoptotic phenotype cells. A strong blue fluorescence indicated a greater degree of typical apoptotic DNA condensation and fragmentation in the nuclei of \textit{C. albicans} cells exposed to I3C than in the intact nuclei of normal control cells. DAPI staining of I3C treated cells showed the distributed nuclear fragments (Fig. 3A-b). Similar results were obtained by TUNEL assay staining of nuclear DNA strand breaks in the late stages of apoptosis. TUNEL-positive cells, which were a strong green fluorescence or intensive green fluorescence spots, were observed in the population treated with I3C (Fig. 3B-b). However, cells treated with thiourea or trehalose are showed as a not treated control cells. In untreated cultures, the nucleus appears as a single round spot in the cell (Fig. 3A-a) or did not show up well against backgrounds (Fig. 3B-a). These analyses presented one of the late stages apoptosis, such as nuclear DNA condensation and fragmentation. Supporting our observations, exposure of \textit{C. albicans} cells to I3C resulted in apoptotic DNA damage, which is considered an important phenomenon of late stage apoptosis in yeast. Furthermore, we ascertained the oxidative stress protecting effects of thiourea and trehalose.

**Formation of Hydroxyl Radicals** We examined hydroxyl radicals formations with HPF, which is oxidized by hydroxyl radicals with a high specificity, since they have been suggested as a crucial cause of apoptosis in many studies. There was a significant increase of the fluorescence signal when the hydroxyl radical formations were in progress. Consistent with the increase in intracellular ROS, the level of intracellular hydroxyl radicals was markedly increased in I3C treated cells (Fig. 4). These results indicate that hydroxyl radicals, which are considered crucial factors in aging and apoptosis, were caused by I3C. To demonstrate that thiourea has a hydroxyl radical scavenger effects and trehalose has a protective effects on environmental stress, we additionally treated I3C exposed cells with thiourea and trehalose. Thiourea significantly reduced hydroxyl radicals formation in I3C treated cells (Fig. 4B), and there appeared to be protective action of trehalose (Fig. 4A). We used thiourea and trehalose in subsequent experiments to determine the effect of hydroxyl radicals reduction on the mitochondria-mediated apoptosis.

**Cytochrome c Release** Cytochrome \(c\) release from the mitochondria to the cytosol is a typical feature of apoptotic cell death. The cytochrome \(c\), which is located in the mitochondrial membrane, is released into the cytosol at the early apoptosis, and then caspase-cascade is activated as a representative of the other apoptotic proteases. Cytochrome \(c\), a soluble protein electrostatically bound to the outer face of the inner mitochondrial membrane, is an essential component of the respiratory chain acting as an electron carrier. As a result of the mitochondrial electron transport system defect, the cytochrome \(c\) is reduced when it release to the cytosol. In this regard, we investigated whether I3C treated cells could induce cytochrome \(c\) release from mitochondria. The amount of cytochrome \(c\) was detected in the cytosolic buffer medium following I3C treated cells, while the cytochrome \(c\) rarely appeared in the supernatants additionally treated with thiourea or trehalose (Fig. 5A). These results show that I3C induces the release of cytochrome \(c\) from mitochondria and suggest that the mitochondria of I3C treated cells, which was suppressed with hydroxyl radicals formations of thioure or trehalose, were not directly affected by the hydroxyl radicals.

**Measurement of Mitochondrial Membrane Potential** The release of cytochrome \(c\) requires an increase in the mitochondrial outer membrane permeability. The in-
Fig. 4. Flow Cytometric Analysis of Hydroxyl Radicals Formation in *C. albicans* upon Exposure to I3C (A, B), AMB (C) and H$_2$O$_2$ (D) Using the Dye HPF

In each panel, thin black lines represent a not treated control and solid black lines represent individual only I3C, AMB or H$_2$O$_2$. I3C treat with trehalose (gray) and with thiourea (gray). An increase of fluorescent signal (shift to the right) corresponds with the formation of hydroxyl radicals.

Fig. 5. Detection of Cytochrome c Releases from *C. albicans* Mitochondria Following Incubation with I3C

Cytosol was ultra centrifuged, and the supernatants were subjected to SDS-PAGE and Western blotting using released cytochrome c (A). Loss of the mitochondrial inner membrane potential induced by *C. albicans* were treated with I3C, AMB, and H$_2$O$_2$ (B). In each panel, thin black lines represent an untreated control and solid black lines represent individual only I3C (a, b), AMB (c), or H$_2$O$_2$ (d). I3C treated with trehalose (gray) and thiourea (gray). Cells were stained with DiOC$_6$, and fluorescence was measured by flow cytometry. A decrease of fluorescent signal (shift to the left) corresponds with the loss of mitochondrial membrane potential.
crease of the mitochondrial transmembrane potential, which is predicted to promote an osmotic matrix swelling, is associated with one model for cytochrome \( c \) release from mitochondria during apoptosis. Because the inner membrane of mitochondria, with its numerous cristae, has a considerably larger surface area than the outermembrane, expansion of the inner membrane upon matrix swelling can break the outermembrane, which would be expected to trigger the release of cytochrome \( c \) to the cytosol.\(^{25}\) In many systems, apoptosis is associated with the loss of mitochondrial inner membrane potential (\( \Delta \Psi_m \)), which may be regarded as a limiting factor in the apoptotic pathway. Reduction of the mitochondrial inner membrane potential (\( \Delta \Psi_m \)) is among the changes encountered during early reversible stages of apoptosis and is preceded by cytochrome \( c \) release in several cell types.\(^{26}\) Thus, we finally determined mitochondrial membrane potential (\( \Delta \Psi_m \)).

To investigate whether I3C can induce a decrease in mitochondrial membrane potential (\( \Delta \Psi_m \)), \( \Delta \Psi_m \) was measured using a mitochondria-specific voltage-dependent dye, DiOC\(_6\)(3), which aggregates into healthy mitochondria and fluoresces green. When the mitochondrial membrane depolarizes, the dye no longer accumulates and is instead distributed throughout the cell resulting in a decrease in green fluorescence.\(^{27}\) The results showed that I3C treated cells were decreasing in the \( \Delta \Psi_m \), in accordance with the pattern induced by experimentally applied AMB and \( \text{H}_2\text{O}_2 \) used as a positive control (Fig. 5B). The decrease in the DiOC\(_6\)(3) fluorescent signal could reflect the depolarization of the mitochondrial membrane. Therefore, the results seem to suggest that I3C could induce the breakdown of \( \Delta \Psi_m \) and the loss of mitochondrial permeability. On the other hand, the cells which treated I3C with thiourea or trehalose did not undergo substantial changes (Figs. 5B-a, b). These results seem to indicate the possibility that the restriction of hydroxyl radical formation would help to maintain the balance of the mitochondrial membrane.

**Measurement of Metacaspase Activation** Caspases are typically activated in the early stages of apoptosis and they play a central role in the apoptotic signaling network. Although caspases are not present in fungi, orthologs of caspases in animals, termed metacaspases, have been identified in fungi and plants,\(^{28,29}\) and their activity can be assessed using the same detection marker.\(^{29,30}\) In order to confirm metacaspase activation, cells were incubated with the CaspACE\textsuperscript{TM}, FITC-VAD-FMK In Situ Marker that binds to the active site of metacaspases and detected using flow cytometry or a fluorescence microscope. A flow cytometric analysis of the cells treated with I3C showed a significant green fluorescence increase in the FITC-VAD-FMK-loaded cells that was consistent with the positive control cells treated with AMB and \( \text{H}_2\text{O}_2 \) (Fig. 6A). Additionally, activated metacaspases decreased, which reduced hydroxyl radicals formation in thiourea treated cells (Fig. 6A-b) and protecting environmental stress by trehalose (Fig. 6A-a) as well as expected. These similar results also displayed when we observed with fluores-
cence microscope (Fig. 6B). Cells with intracellular active metacaspases stained green fluorescent, whereas nonapoptotic cells appeared unstained. These results suggest that I3C treatment did initially lead to a significant generation of strong oxidant hydroxyl radicals, which are well-known to be important regulators of yeast apoptosis, and then hydroxyl radicals activated metacaspase.

In conclusion, this study presents the first evidence for I3C promoting apoptosis in \textit{C. albicans} via the intracellular ROS accumulation, phosphatidylserine externalization, DNA damages, and metacaspases activation. Ultimately, I3C induces disruption of the mitochondrial integrity, and cytochrome \textit{c} releases. We also conducted experiments to learn how the thiourea impacts with the hydroxyl radical accumulation. Thiourea is a potent hydroxyl radical scavenger that is an established means of mitigating the effects of hydroxyl radical damage in both eukaryotes and prokaryotes.\textsuperscript{31,32} Interestingly, the results showed that the apoptosis phenomena were inhibited when hydroxyl radicals which were induced from ROS, were removed by thiourea. These results seem to indicate the possibility that hydroxyl radicals, which are created by I3C, contribute to the apoptosis in \textit{C. albicans}. ROS damages iron-sulfur clusters, making ferrous iron available for oxidation by the Fenton reaction, and these events appear to be mediated by the tricarboxylic acid (TCA) cycle and a transient depletion of nicotinamide adenine dinucleotide (NADH).\textsuperscript{33} The Fenton reaction leads to hydroxyl radical formation, and the hydroxyl radicals damage DNA, proteins, and lipids, which results in cell death. Moreover, we examined the tolerance of intracellular trehalose as a stress response on I3C in \textit{C. albicans}. The results showed that trehalose attenuated the apoptosis effects of the I3C during the proceedings. Trehalose is a non-reducing disaccharide that is present in diverse organisms ranging from bacteria and fungi to invertebrates. In yeast, trehalose plays a role in osmotic stress tolerance. It has been shown that trehalose can protect proteins and cellular membranes from inactivation or denaturation caused by a variety of stress conditions including desiccation, dehydration, heat, cold, oxidation, and toxic agents.\textsuperscript{34,35}

Briefly, the intracellular ROS level was elevated including hydroxyl radicals, cytochrome \textit{c} was released, and the mitochondrial membrane potential was changed after exposure to I3C. Several other studies have linked cytochrome \textit{c} release, ROS formation, and changes in mitochondrial membrane potential to yeast apoptosis.\textsuperscript{36} These support that I3C has a damaging effect on the mitochondria. Additionally, we gathered from the way apoptotic phenomena was reduced by oxidative stress protecting substances that the apoptotic cell death induced by I3C is related with ROS. Thus, the findings herein indicate I3C triggers apoptosis in \textit{C. albicans} through generation of reactive oxygen species and then formation of hydroxyl radicals, which deserves further studies to define apoptosis mechanisms of I3C.

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