We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900
Open access books available

185,000
International authors and editors

200M
Downloads

Our authors are among the

154
Countries delivered to

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

Chapter 4

Differential Effect of Atpenin A5 on ROS Production from Wild-Type Mitochondrial Complex II in Human Cancer Cells and Normal Cells

Madhavi P. Paranagama and Kiyoshi Kita

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.71638

Abstract

Human mitochondrial complex II is an intriguing enzyme, which has been the focus of medical research during the past few decades since it contributes to pathogenesis of mitochondrial diseases as well as a target for chemotherapy. Reactive oxygen species (ROS) produced by this enzyme has been implicated in both these conditions. While ROS produced from mutated mitochondrial complex II has been implicated in pathogenesis of mitochondrial diseases, ROS produced from pharmacologically inhibited mitochondrial complex II has been implicated in cancer cell death. In this chapter, we show that inhibition of mitochondrial complex II in human cancer cells with atpenin A5 produces detectable levels of ROS while normal cells do not. Thus, this enzyme may be used as a potential target for developing new anticancer drugs to trigger ROS-mediated selective death of cancer cells.

Keywords: mitochondrion complex II, ROS, anticancer agents, atpenin A5

1. Introduction

Human mitochondrial complex II (succinate:ubiquinone oxidoreductase; succinate dehydrogenase (SDH)) is an intriguing enzyme, which has been the focus of medical research during the past few decades since it contributes to pathogenesis of mitochondrial diseases (for recent reviews, see [1, 2]) and also a target for chemotherapy [3–5].

1.1. Structure of mitochondrial complex II

Mitochondrial complex II is a heterotetrameric protein embedded in the inner mitochondrial membrane. Its four subunits are referred as flavoprotein subunit (Fp), iron-sulfur subunit

IntechOpen

© 2018 The Author(s). Licensee IntechOpen. This chapter is distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. CC BY

(Ip), cytochrome *b* large subunit (CybL), and cytochrome *b* small subunit (CybS). The Fp and Ip subunits comprise the catalytic domain of the enzyme. The Fp subunit has a flavin adenine dinucleotide (FAD) as a prosthetic group and contains the dicarboxylate-binding site, which serves as the binding site for succinate and fumarate. The Ip subunit generally contains three iron-sulfur clusters $[2Fe\text{-}2S]^{2+,1+}$, $[4Fe\text{-}4S]^{2+,1+}$, and $[3Fe\text{-}4S]^{1+,0}$ as prosthetic groups. Subunits CybL and CybS, with heme *b* as the prosthetic group, form the anchor domain of the enzyme. This anchors the catalytic domain to the inner mitochondrial membrane and also forms the quinone (Q)-binding site, together with the Ip subunit [6, 7] (**Figure 1**).

In 2003, our laboratory revealed that human mitochondrial complex II exists in two isoforms, which differ in the Fp subunit. These two human Fp subunits, which are referred as Fp I and Fp II, differ only in two amino acids in the C-terminal of the protein-Tyr629Phe and Val657Ile [8, 9]. The majority of human tissues have shown expression of both isoforms of complex II with predominant expression of type I Fp. However, some cancer cell lines and fetal tissues have shown predominant expression of type II Fp also [4]. Furthermore, type II Fp expression has been found to increase in cultured cells under ischemic conditions [10]. Moreover, our laboratory has revealed that Fp subunit of human complex II undergoes posttranslational modifications by phosphorylation of its tyrosine, threonine, and serine residues under tumor mimicking microenvironments (hypoxic and hypoglycemic conditions), which affects its enzymatic activity [11].

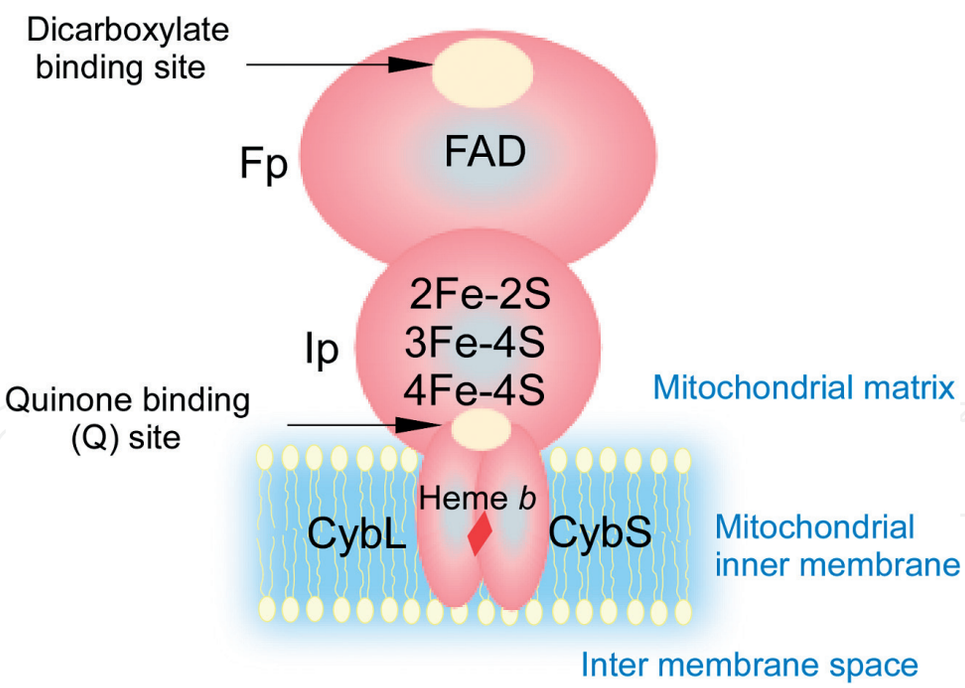


Figure 1. Schematic representation of the mitochondrial complex II. The four subunits of the complex II, namely the flavoprotein subunit, iron sulfur subunit, cytochrome *b* large subunit, and cytochrome *b* small subunit are labeled as Fp, Ip, CybL, and CybS, respectively. The prosthetic groups that participate in the electron transfer are the flavin adenine dinucleotide (FAD), three iron sulfur clusters 2Fe-2S, 3Fe-4S, 4Fe-4S, and heme *b*. Dicarboxylate-binding site in the Fp subunit serves as the catalytic site for succinate oxidation/fumarate reduction. Quinone-binding site comprised of Ip, CybL, and CybS subunits serves as the catalytic site for quinone reduction/quinol oxidation.

http://dx.doi.org/10.5772/intechopen.71638

1.2. Function of mitochondrial complex II

In human cells, this enzyme plays dual roles as an indispensable enzyme in the TCA cycle as well as in the aerobic respiratory chain (**Figure 2**). It oxidizes succinate to fumarate in the TCA cycle and reduces quinone to quinol in the aerobic respiratory chain [6, 12]. This is in contrast to the function of the mitochondrial complex II of the anaerobic organisms such as *Ascaris suum*, which oxidizes quinol into quinone that is coupled with reduction of fumarate to succinate (**Figure 3**) [13]. However, our laboratory has revealed distinct fumarate reductase (FRD) activity in the human cancer cells, which have significantly increased upon exposure to hypoxia and hypoglycemia. In these cells, mitochondrial complex II is reported to acquire FRD activity through phosphorylation of its Fp subunit [11]. This finding is further supported

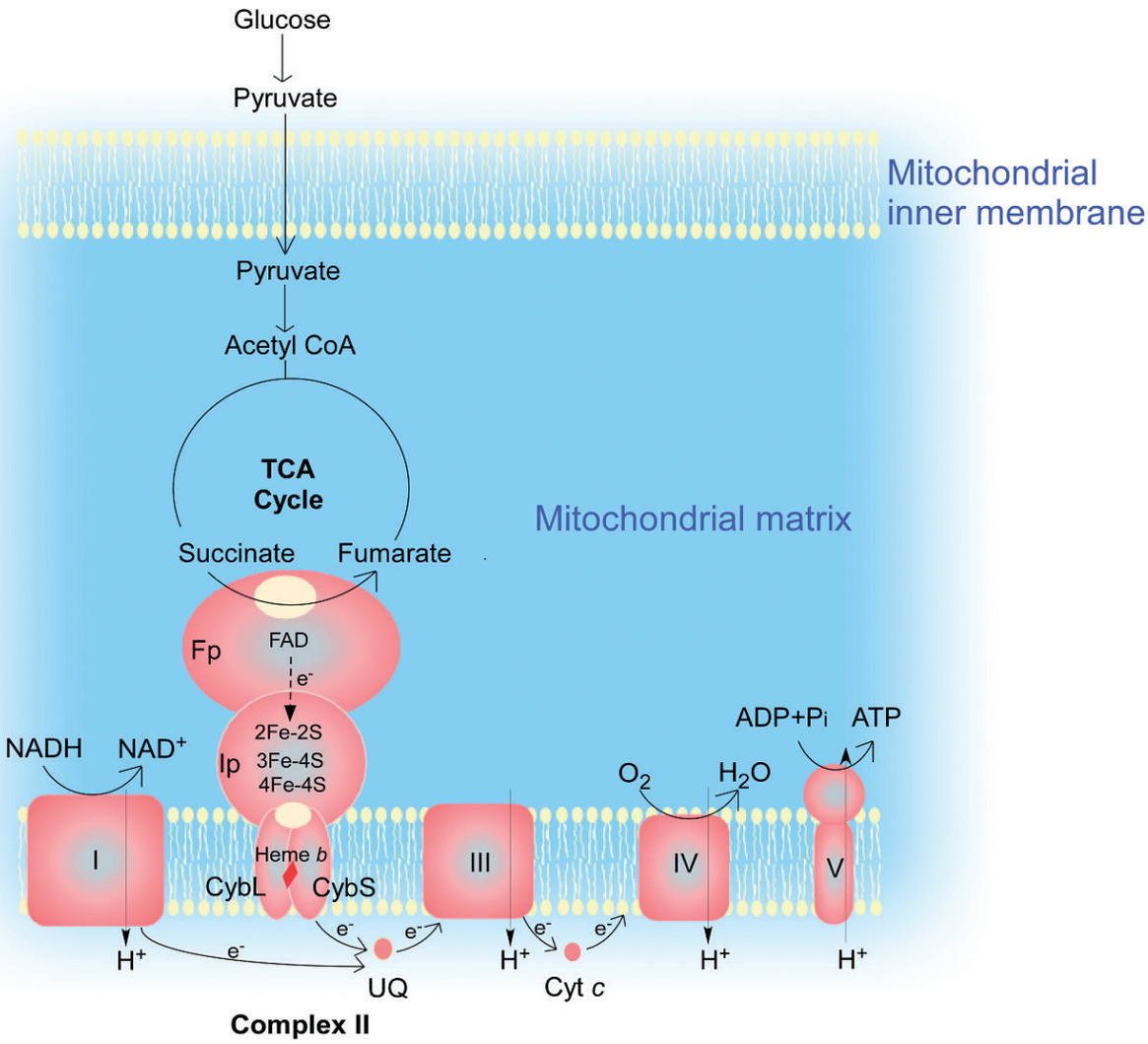


Figure 2. Schematic representation of the function of the mitochondrial complex II in the aerobic respiratory chain and the TCA cycle. Functioning as a component of the TCA cycle, mitochondrial complex II catalyzes the oxidation of succinate to fumarate with transfer of electrons to FAD. Functioning as a component of the aerobic respiratory chain, the electrons on FAD are transferred through Fe-S clusters and heme *b* to reduce the ubiquinone. For simplicity of the representation, the subunit composition of the respiratory chain complexes I, III, IV, and V is not shown.

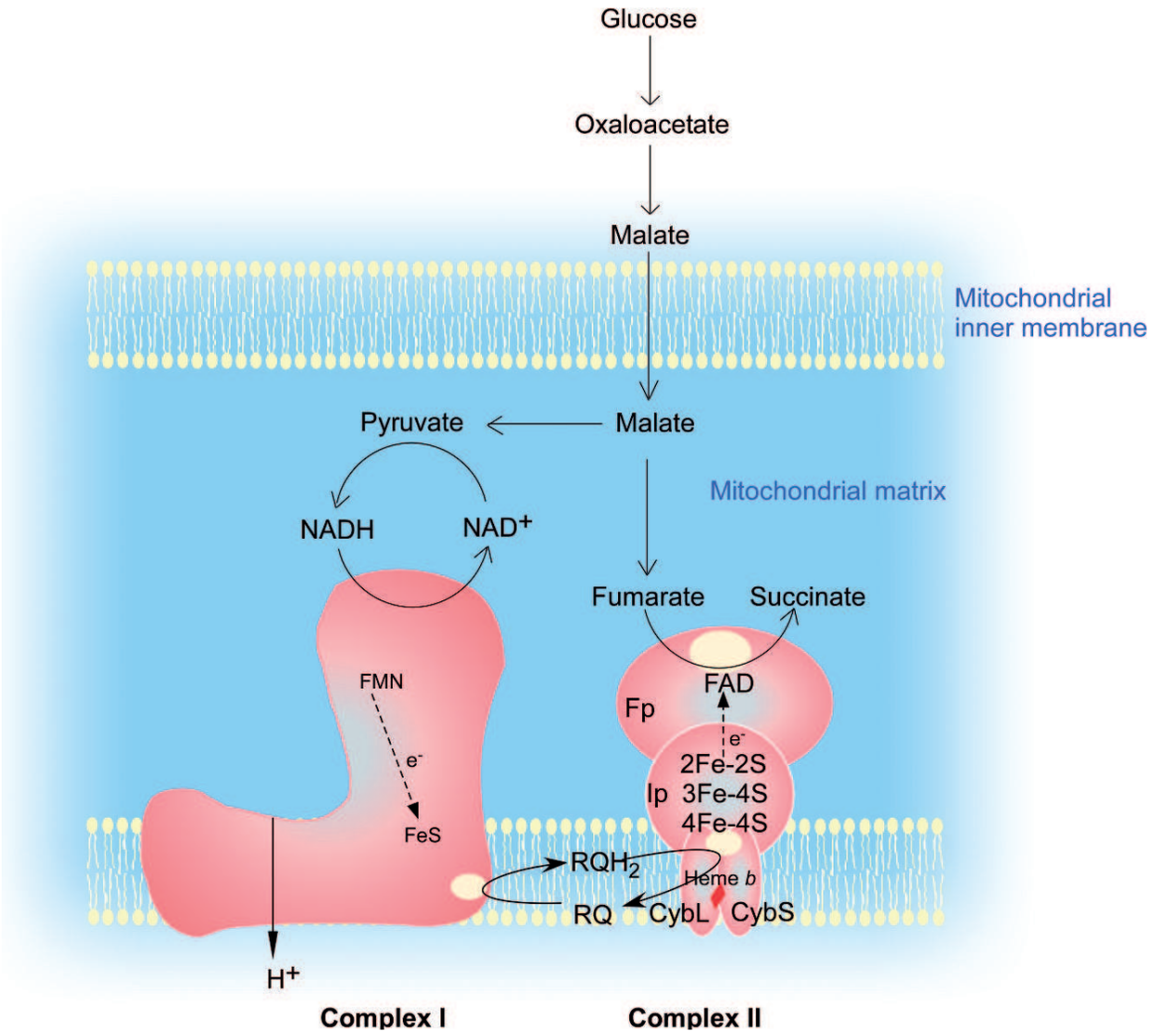


Figure 3. A schematic representation of the function of the mitochondrial complex II in the anaerobic respiratory chain. Electrons originating from NADH oxidation in complex I reduce the rhodoquinone (RQ) to rhodoquinol (RQH2) at its Q site. Rhodoquinol is subsequently oxidized to rhodoquinone at the Q site of the complex II. The resulting electrons are transferred via the Fe-S clusters and FAD to reduce fumarate to succinate. Fumarate is the terminal electron acceptor of the anaerobic respiratory chain.

by recent metabolic profiling studies also on gastric and colon carcinomas [14]. In addition to its role in energy metabolism, mitochondrial complex II has been identified to have a novel function as a tumor suppressor in certain tissues. Mutations in its subunits are reported to cause cancers such as pheochromocytoma (tumors of the chromaffin cells in the adrenal medulla) and paraganglioma (extra adrenal tumors of sympathetic or parasympathetic origin). Moreover, breast, thyroid, and renal carcinomas are also known to be associated with complex II mutations. More recently, mutations in the complex II assembly factors, succinate dehydrogenase assembly factor 1 (SDHAF1) and succinate dehydrogenase assembly factor 2 (SDHAF2), are also reported to be linked with infantile leukoencephalopathy and paraganglioma (for a recent review, see [2]).

http://dx.doi.org/10.5772/intechopen.71638

1.3. ROS production from mitochondrial complex II

Reactive oxygen species (ROS) are inevitable byproducts of the aerobic respiratory chain. Major ROS produced by the respiratory chain is superoxide (O_2^-). It is subsequently dismutated into hydrogen peroxide (H_2O_2) by superoxide dismutase (**Figure 4**). Both O_2^- and H_2O_2 are known to contribute to physiological and pathological redox signaling [15, 16].

However, conventionally, mitochondrial complex II is not identified as a significant source of ROS. But, two potential ROS-producing sites can be identified in mitochondrial complex II when its sequence of electron transport is considered together with the spatial arrangement of its redox centers. When one molecule of succinate is oxidized by complex II, FAD is reduced with two electrons, but subsequent transfer of electrons through Fe-S clusters occurs one at a time. Hence, when one electron is delivered to the proximal Fe-S cluster from FAD, a flavin radical is formed until the second electron is delivered. Similarly, after sequential transfer of single electrons through Fe-S clusters, quinone is reduced with two electrons. Hence, when one electron arrives at the Q site, until another electron arrives from Fe-S clusters, a quinone radical is formed. These two radicals have the potential to generate ROS, if there is accessibility to oxygen (**Figure 5**).

During the past two decades, complex II has emerged as an important source of ROS. On the one hand, ROS produced from mutated complex II has been proposed to be the underlying cause of complex II–associated human diseases. Thus, numerous studies have been conducted to understand ROS production from human complex II. Most of these studies have been conducted on model organisms such as *Escherichia coli, Saccharomyces cerevisiae, and Caenorhabditis elegans* [17–20]. A hallmark in these studies is the finding that the *mev-1* mutation in the mitochondrial complex II of *C. elegans* results in enhanced O_2^- production and oxygen hypersensitivity [19, 20]. Later on, this finding was further reinforced by a study using a transgenic mouse cell line with the equivalent mutation in the *CybL* gene as the *mev-1* mutation demonstrating O_2^- overproduction from mitochondria that leads to tumorigenesis [21]. More recently, mutations in the Ip subunit in the *C. elegans* have also been found to display enhanced ROS production [22]. Site-directed mutagenesis of CybS subunit in *E. coli* [23] and the Ip and CybS subunits in *S. cerevisiae* [18, 24] has also shown enhanced ROS production providing further support for the hypothesis that ROS underlies the complex II mutations-associated carcinogenesis. Indeed, some studies have been conducted on human cell lines using pharmacological and genetic interventions to mimic complex II mutations, but the results are controversial. For example, ROS production from human mitochondrial complex II has been shown by Guzy et al. [25] in intact cells of the hepatoma cell line Hep3B by inhibiting the complex II activity by the Q site inhibitor theonyl trifluoroacetone (TTFA) and by RNA interference of Ip subunit. In contrast, RNA interference of the same subunit of complex II in the same cell line by Cervera et al. have failed to detect ROS production [26].

On the other hand, ROS produced from mitochondrial complex II has received considerable attention as a mediator of "mitocans" (a novel class of mitochondrial targeted anticancer drugs) induced cancer cell death [5]. Interestingly, complex II Q site inhibiting mitocan, α-tocopheryl succinate (α-TOS) has been found to induce ROS only in complex II competent

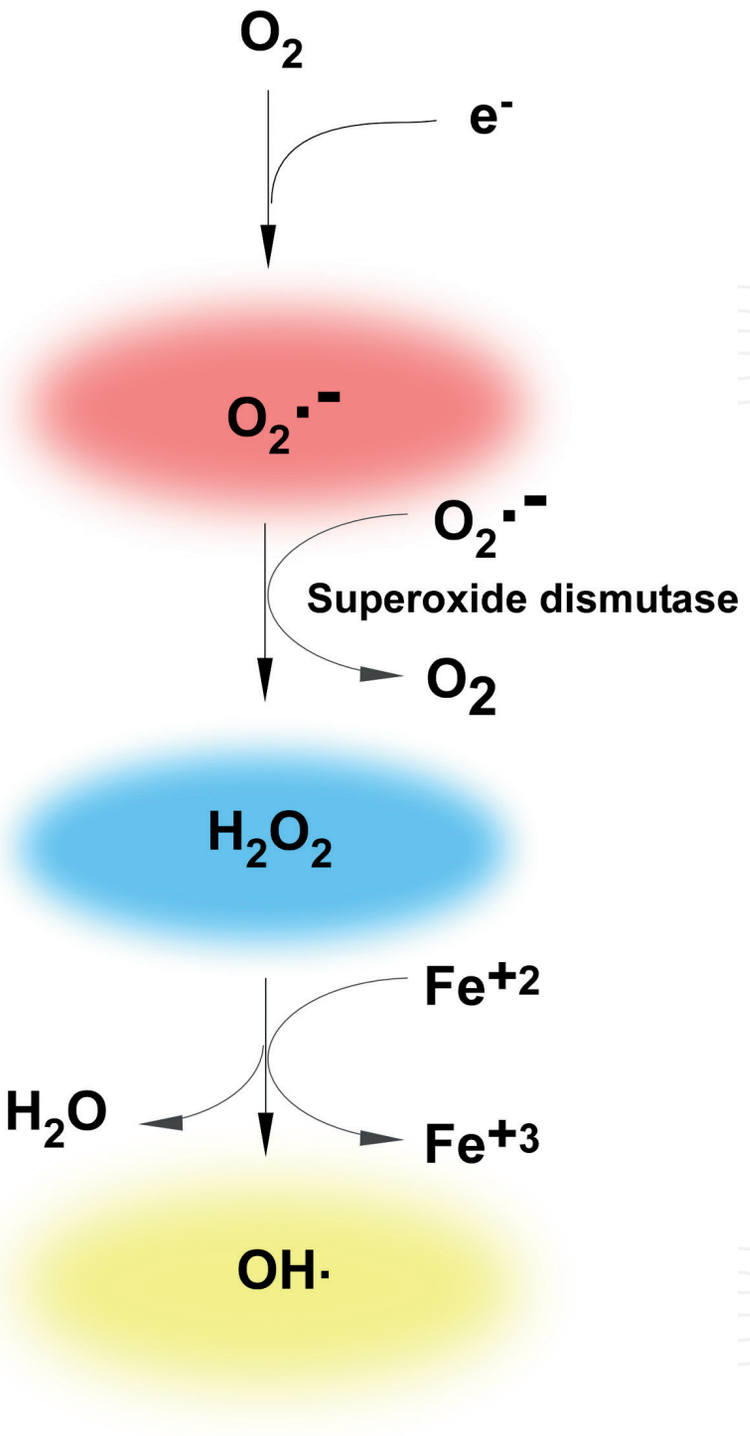


Figure 4. Diagrammatic representation of the production of the reactive oxygen species (ROS). When oxygen is reduced with a single electron, O_2^- is formed. Superoxide is converted to H_2O_2 by the enzyme superoxide dismutase. In the presence of transition metals such as Fe^{2+}, H_2O_2 is converted into hydroxyl radicals ($OH^{\bullet}$) by the Fenton reaction.

cells but not in complex II mutant cells [27, 28], indicating the importance of a wild-type complex II for its action. More importantly, this complex II inhibitor has caused selective death of cancer cells, but the exact reason for this selectivity is not yet understood [29].

http://dx.doi.org/10.5772/intechopen.71638

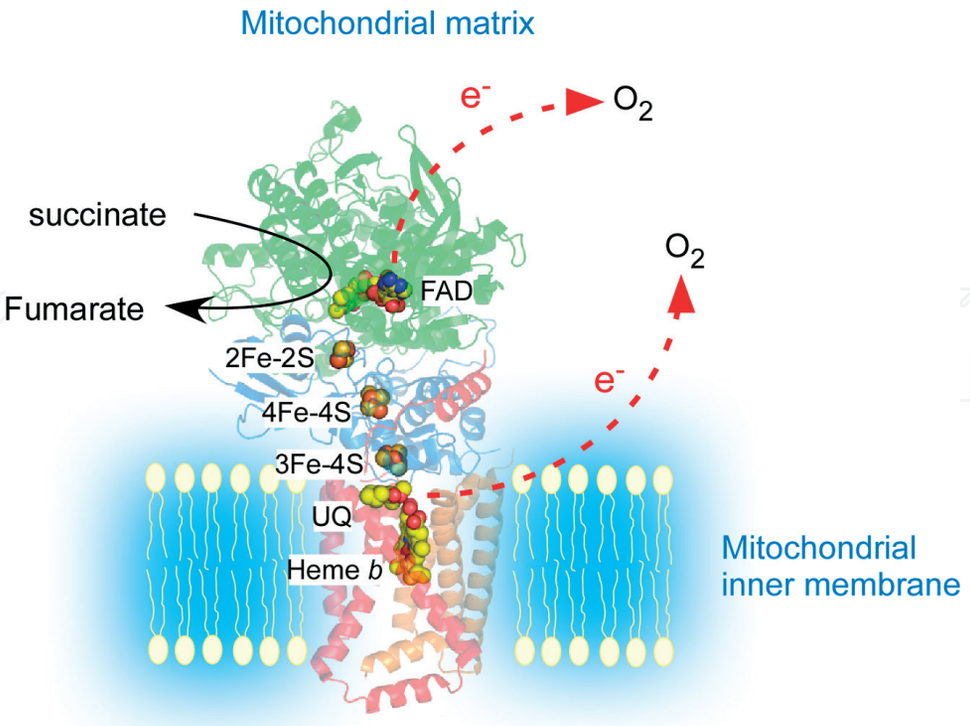


Figure 5. Ribbon model of mitochondrial complex II of *Sus scrofa* (pdb1ZOY) showing the potential ROS production sites. Fp, Ip, Cy*b*L, and Cy*b*S subunits are shown in green, blue, red, and orange, respectively. The redox centers—FAD, iron sulfur clusters, and heme *b*—are labeled in the figure.

In this chapter, we show that the potent and specific complex II Q site inhibitor atpenin A5 [30] can specifically induce ROS production in two human cancer cell lines HT-29, which predominantly express Fp I, and DLD-1, which predominantly express Fp II in the mitochondrial complex II but not in the noncancerous tissue, human dermal fibroblasts that predominantly express Fp I. This difference in ROS production may be attributed to the difference in the post-translational modifications of the wild-type mitochondrial complex II's in normal cells and cancer cells. This finding highlights new avenues in developing complex II-targeted mitocans with considerable promise.

2. Materials and methods

2.1. Culture of cells

Human colon cancer cell lines HT-29, which predominantly express Fp I in its complex II, and DLD-1, which predominantly express Fp II in its complex II, were obtained from ETCC and grown in glucose-free Roswell Park Memorial Institute (RPMI) medium supplemented with 1 g/L of glucose with 10% heated fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B at 37°C and 5% CO_2 in a humidified incubator. The medium was renewed in 48 h intervals, and the cells were harvested at 70% confluence.

Human dermal fibroblasts, which predominantly express Fp I in its complex II, were obtained from Cell Applications Inc., Japan, and grown in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM-F12).

2.2. Analysis of complex II isoform expression

Analysis of complex II isoform expression was carried out as described previously [8]. Briefly, total RNA was isolated from cultured cells using TRIzol LS reagent (Invitrogen) according to the manufacturer's instructions. After removal of residual DNA by treating with DNAse, cDNA was synthesized from total RNA using ReverTraAce (Toyobo, Japan) and an oligo (dT) primer. DNA sequence of the Fp I subunit of the complex II was amplified using Taq DNA polymerase with the sense primer 5′tacggagcaggca 3′ and the antisense primer 5′aatggtggcgggac 3′ and that of the Fp II subunit was amplified with the sense primer 5′cggacagaggcg3′ and the antisense primer 5′aatggctggcgggat3′. The resulting PCR products were subjected to agarose gel electrophoresis, and the two isoforms were identified by the size of the PCR products in the ethidium bromide-stained agarose gels scanned with the electronic transilluminator FAS III (Toyobo, Japan).

2.3. Isolation of mitochondria

Cells cultured in three 225 cm^2 flasks were harvested by trypsinization. The cells were suspended in mitochondria preparation buffer (250 mM sucrose, 20 mM HEPES, 3 mM EDTA, and 1 mM sodium malonate), pH 7.5, and homogenized with a power-driven glass-teflon Potter-Elvehjem homogenizer (20 passes). The homogenate was centrifuged at 700 × g for 15 min to pellet the cell debris and nuclei. The resulting supernatant was centrifuged at 14,000 × g for 15 min to sediment the mitochondria. The pellet was washed thrice with the mitochondria isolation buffer without sodium malonate, resuspended in the same buffer, and stored on ice until analysis. Integrity of the mitochondria was analyzed by citrate synthase activity in the presence and absence of 0.05% sucrose monolaurate (SML) in the assay mixture.

2.4. H_2O_2 assay in isolated mitochondria

Hydrogen peroxide-mediated oxidation of the fluorescent probe Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) to resorufin was used to determine the H_2O_2 generation rate of the isolated mitochondria as described previously [31, 32]. Since Amplex Red does not enter the mitochondria, polyethylene glycol-conjugated SOD (PEG-SOD), a membrane permeable form of SOD, was added to the reaction mixture to convert the O_2^- produced in the mitochondrial matrix into H_2O_2, which is subsequently released to the cytosol. The assay was performed in a 96-well plate maintained at 25°C. The resorufin formation rate was measured in SpectraMax Plus spectroflourometer (Molecular Devices, Sunnyvale, CA) at an excitation wavelength of 540 nm and emission wavelength of 590 nm. The H_2O_2 generation rate was calculated using a standard curve (0–1000 nmol/ml). To eliminate the fluorescence increment due to nonspecific oxidation of Amplex Red, a parallel assay was performed in the presence of catalase, an enzyme that scavenges H_2O_2, and the result was obtained by subtracting the amount of H_2O_2 produced in the presence of catalase from that in the absence of catalase.

H_2O_2 generation assays were also performed in the presence of respiratory chain inhibitors-10 mM nitropropionic acid (NPA), which blocks the dicarboxylate-binding site of complex II; 10 μM atpenin A5, which blocks the Q site of complex II; and 10 μM antimycin A, which blocks the Qi site of complex III in order to dissect the specific ROS-producing site of the respiratory chain.

2.5. Superoxide assay in intact cells

MitoSOX Red is a fluorescent probe that is targeted to the mitochondria of intact cells and oxidized by O_2^- produced within mitochondria. This probe was used to detect O_2^- production in the intact cells as previously described with slight modifications [33, 34]. Briefly, DLD-1 and HT-29 colon epithelial cells and the human dermal fibroblasts were cultured on cover slips and loaded with 5 μM MitoSOX Red and 50 nM MitoTracker Green (Molecular Probes, Eugene, OR) in the dark at 37°C in the CO_2 incubator for 15 min. After washing the cells for three times with prewarmed phosphate-buffered saline (PBS), three cover slip cultures each from DLD-1 and HT-29 cells were treated with the vehicle (0.2% DMSO), PEG-SOD or 10 mM NPA and incubated for 30 min. Thereafter, 10 μM atpenin A5 was added to all the cover slip cultures and imaged at 488 nm/455 nm excitation and emission wavelengths for MitoTracker Green and 633/650 nm for MitoSOX Red immediately (0 min images) and after 30 min (30 min images), using a confocal laser scanning microscope (LSM 510, Carl Zeiss, Germany). The two cover slip cultures of dermal fibroblasts were treated with 0.2% DMSO for 30 min, and subsequently one was treated with 10 μM atpenin A5 and the other one was treated with 1 μM antimycin A. Image acquisition of dermal fibroblasts was done similar to those of DLD-1 and HT-29 cells.

2.6. Statistical analysis

Results of the H_2O_2 assay are expressed as means ± SEM of three independent experiments. The data were analyzed using Shapiro-Wilks test followed by one way ANOVA. Subsequently, Levene's test and Tuckey's post hoc tests were performed. The level of significance was $P < 0.05$. Graph Pad Prism software (version 7, USA) was used for the analysis.

3. Results

3.1. Complex II isoforms in cell lines

cDNA from the two cancer cell lines (HT-29 and DLD-1) and the normal cell line (dermal fibroblasts) were amplified using Fp I- and Fp II-specific primers to analyze the degree of expression of the two Fp types in complex II isoforms. As shown in **Figure 6**, the results revealed that HT-29 and DLD-1 cells predominantly express Fp I and Fp II, respectively, in their complex II, whereas dermal fibroblasts predominantly express Fp I.

3.2. H_2O_2 production from the complex II in isolated mitochondria

As shown by the first bar of **Figure 7a–c**, mitochondria isolated from all three cell lines generated H_2O_2 when incubated with succinate in the absence of any respiratory chain inhibitors. This result

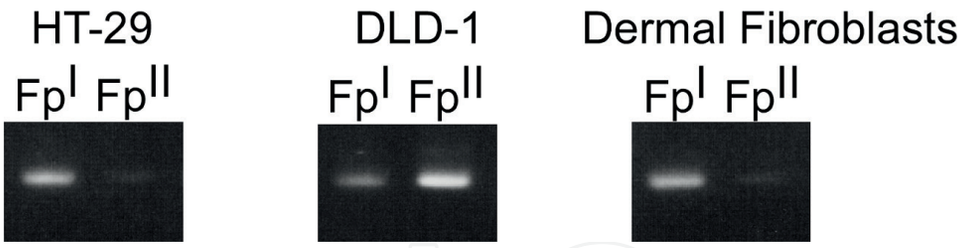


Figure 6. Mitochondrial complex II isoforms expression pattern in HT-29, DLD-1, and dermal fibroblasts. Ethidium bromide-stained agarose gels show the RT-PCR products obtained with Fp-specific primers using the RNA isolated from HT-29, DLD-1, and dermal fibroblasts. Isoform I is comprised of Fp I and isoform II is comprised of Fp II.

shows that mitochondrial complex II in all three cell lines under investigation can rapidly oxidize succinate and feed electrons to their respiratory chains and a portion of those electrons are leaked to O_2 to generate ROS.

The second bar in **Figure 7a–c** shows that addition of atpenin A5, an inhibitor of the Q-binding site of complex II, increases the H_2O_2 production in mitochondria isolated from HT-29 and DLD-1 cells but not in the mitochondria isolated from dermal fibroblasts. It shows that when electron flow through the Q-binding site is blocked, increase in the proton motive force in the respiratory chain proximal to the complex II Q site leads to leakage of electrons from the respiratory chain to oxygen in mitochondria isolated from cancer cells but not in mitochondria isolated from normal cells. This observation implies that a site proximal to the Q-binding site of complex II is responsible for leakage of electrons in cancer cell respiratory chains, which is different from that in normal cells. As shown by the third bar of the **Figure 7a–c**, addition of NPA, an inhibitor of the dicarboxylate-binding site of the mitochondrial complex II, along with atpenin A5 almost completely abolished the succinate-dependent H_2O_2 production in all three cell types. According to this observation, the increment in H_2O_2 production from the mitochondria isolated from cancer cells in the presence of atpenin A5 is attributed to a leakage of electrons from a site upstream of the Q site and downstream of the dicarboxylate-binding site. However, one can argue that the difference observed in H_2O_2 production between mitochondria of cancer cells and normal cells in the presence of atpenin A5 is due to the difference in the antioxidant defense systems between the two cell types. Therefore, we observed the H_2O_2 production in isolated mitochondria in the presence of antimycin A, an inhibitor of the Qi site of complex III that is reported to induce ROS generation from the respiratory chain complex III [32]. As shown by the fourth bar of **Figure 7a–c**, addition of antimycin A to the reaction mixture could increase the H_2O_2 production from mitochondria isolated from cancer cells as well as from the normal cells, indicating that the difference in the antioxidant levels is not the case.

3.3. Superoxide production from the complex II in live cells

When HT-29 and DLD-1 cells grown in RPMI medium containing glucose were treated with atpenin A5, their confocal images showed a clear red fluorescence in the cytoplasm while the untreated cells were devoid of such red fluorescence. To verify the localization of this fluorescence to mitochondria, the cells were simultaneously loaded with MitoTracker Green, a

http://dx.doi.org/10.5772/intechopen.71638

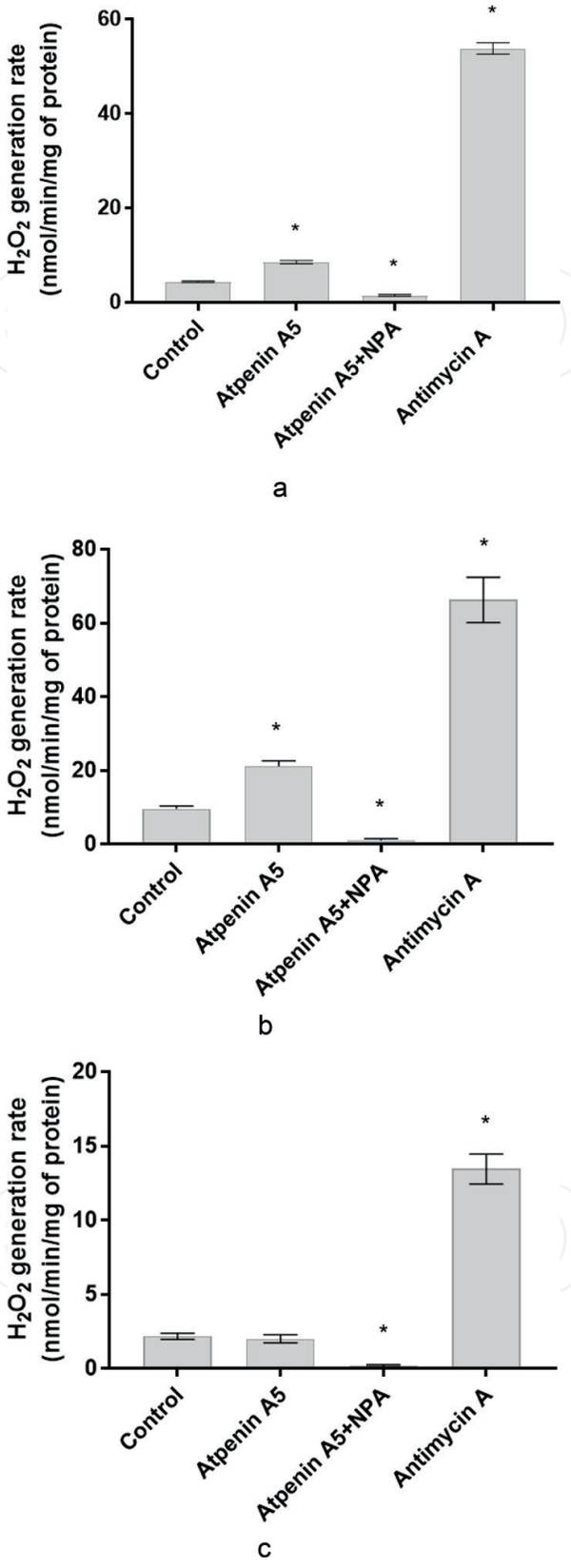
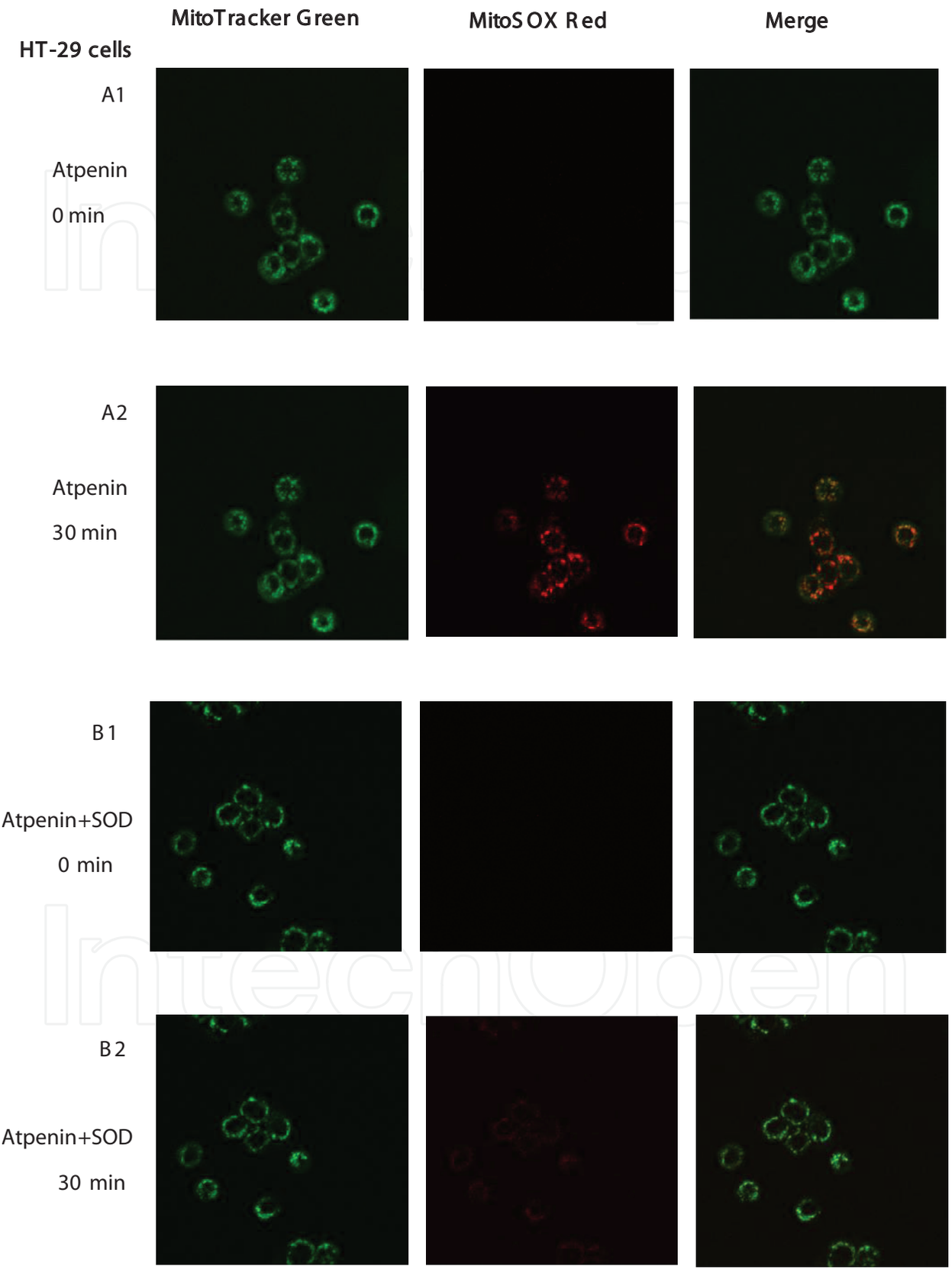
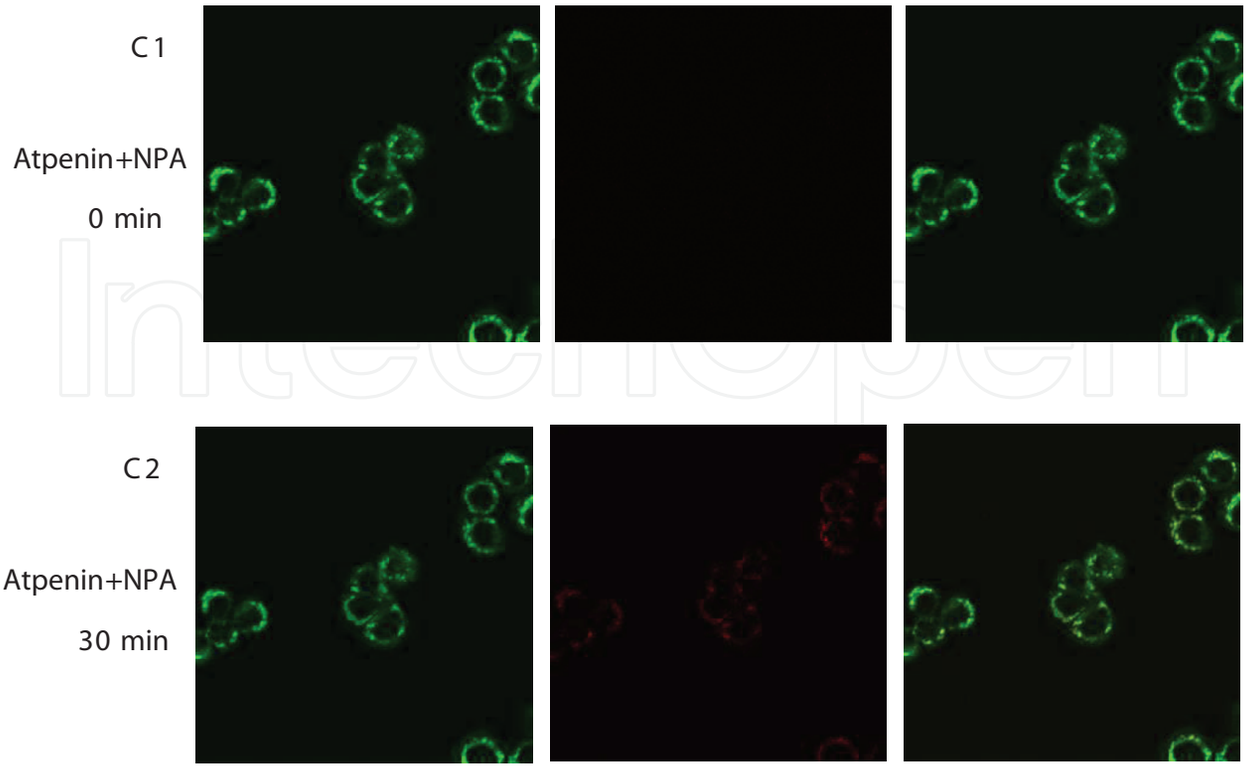
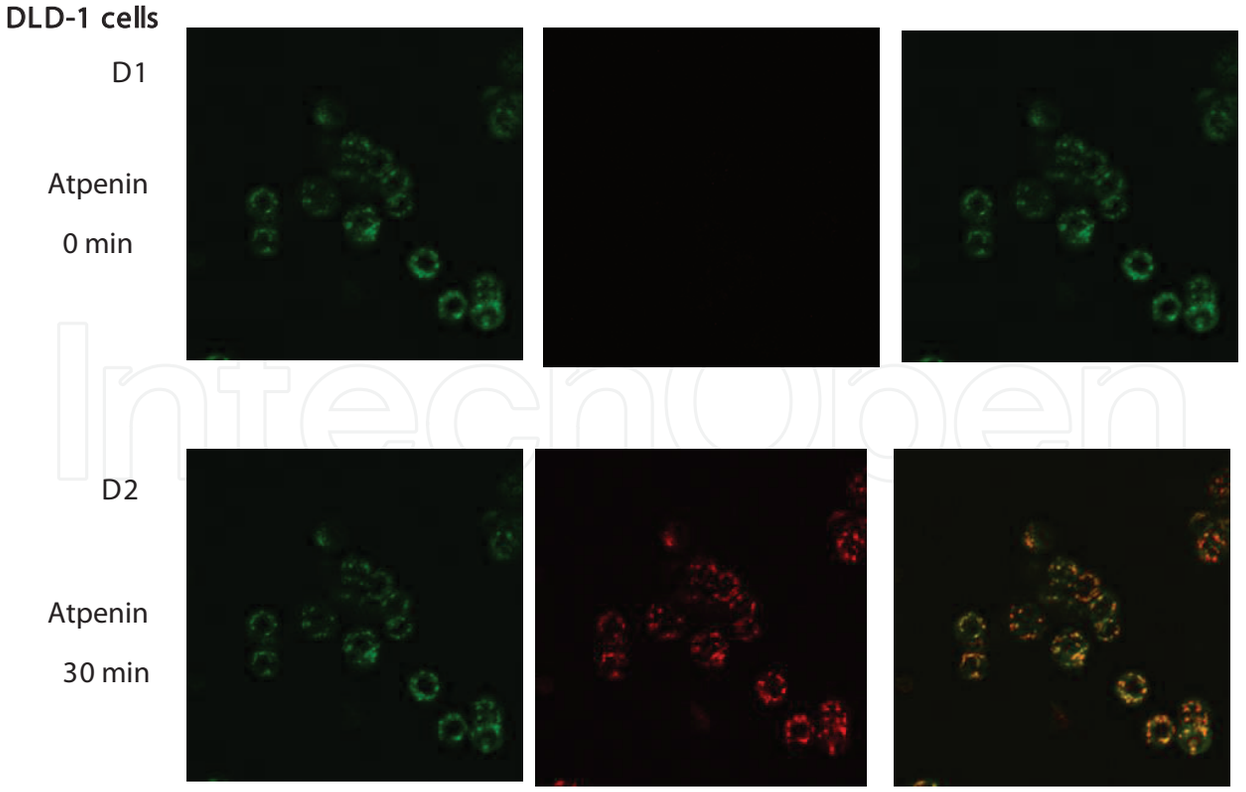


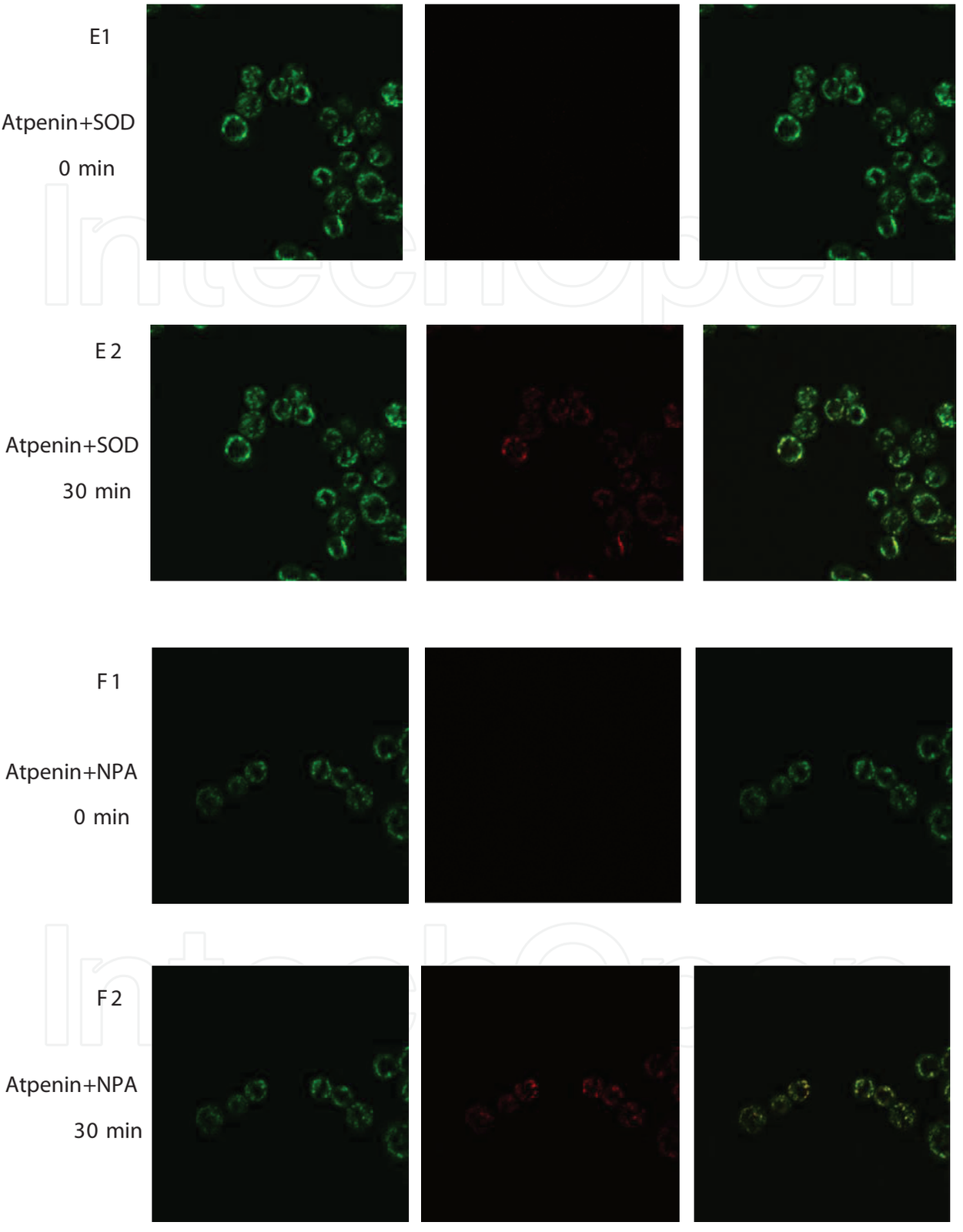
Figure 7. H_2O_2 production from the mitochondria isolated from HT-29 cells (a), DLD-1 cells (b), and dermal fibroblasts (c) oxidizing 0.75 mM succinate. Results are expressed as mean ± SEM of three independent experiments. *Significantly different from control cells ($P < 0.05$).


MitoTracker Green
MitoSOX Red
Merge
HT-29 cells
A1
Atpenin
0 min
A2
Atpenin
30 min
B1
Atpenin+SOD
0 min
B2
Atpenin+SOD
30 min

http://dx.doi.org/10.5772/intechopen.71638






E1
Atpenin+SOD
0 min
E 2
Atpenin+SOD
30 min
F 1
Atpenin+NPA
0 min
F 2
Atpenin+NPA
30 min

http://dx.doi.org/10.5772/intechopen.71638

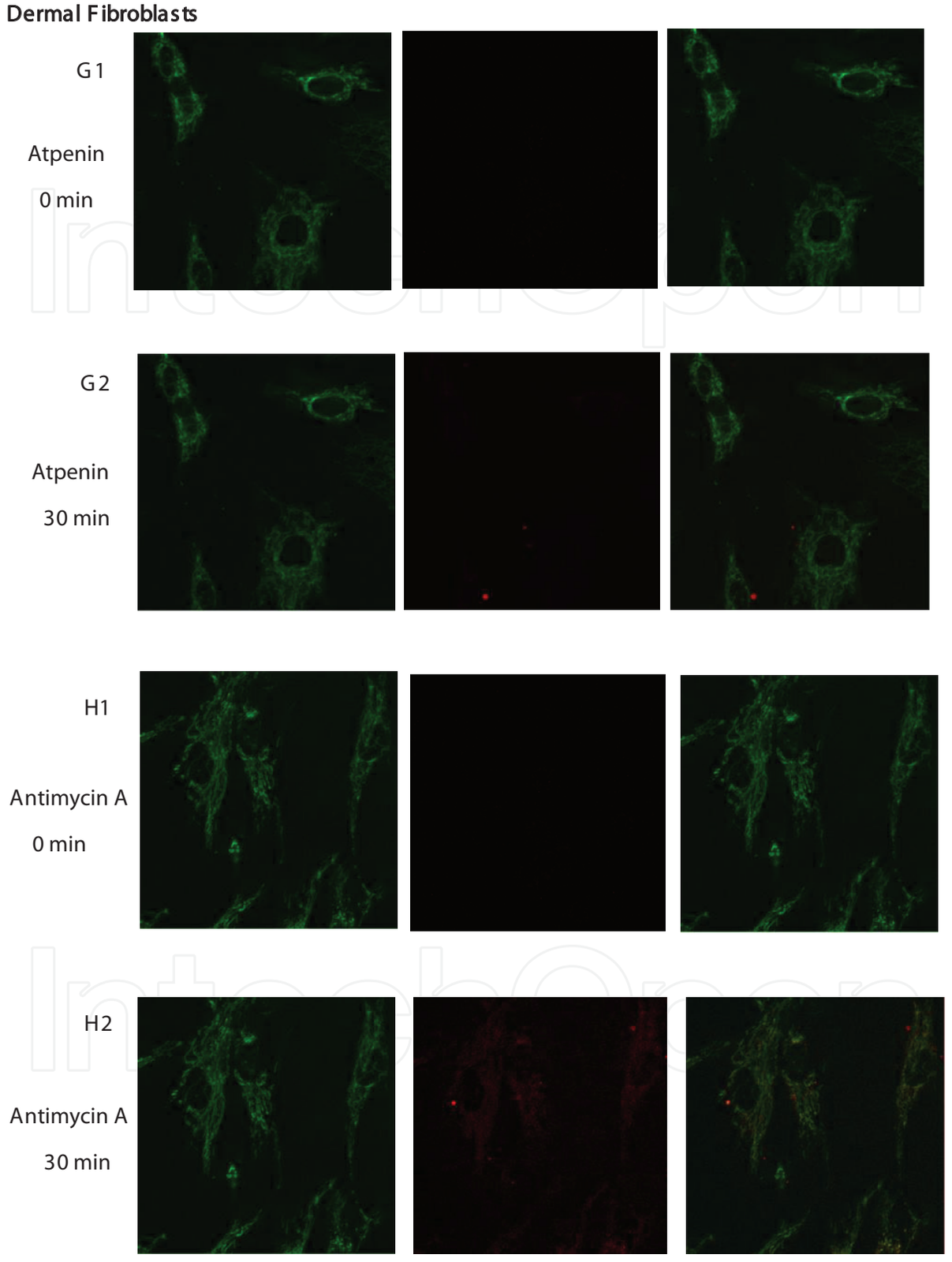


Figure 8. Confocal laser scanning microscopic images showing the O_2^- production from the mitochondrial complex II in live cells. Panels A, D, and G show the time lapse images of the cells preincubated with DMSO and treated with atpenin A5. Panels B and E show the cells preincubated with SOD-PEG and treated with atpenin A5. Panels C and F show the cells preincubated with NPA and treated with atpenin A5. Cells treated with antimycin A are shown in panel H. The cells in the first column have a green florescence. The cells in the second column have a red florescence. Out of the cells in the third column, A2, D2 and H2 panels have a dark orange florescence and B2, C2 and E2 panels have a light orange florescence and other panels have a green florescence.

fluorescent probe that is specifically targeted to mitochondria. The resulting confocal images revealed that the green fluorescence of MitoTracker Green colocalize with the red fluorescence of MitoSOX Red indicating that MitoSOX Red is oxidized within mitochondria (**Figure 8**). To determine whether MitoSOX oxidation is a superoxide-dependent process, the cells were pre-incubated with PEG-SOD, which can scavenge O_2^- generated within cells. In the presence of PEG-SOD, MitoSOX Red fluorescence was negligible (**Figure 8**, panels B and E). This result indicates that the majority of the atpenin A5-induced MitoSOX fluorescence is superoxide dependent. Next, we attempted to determine whether the atpenin A5-induced O_2^- production is a succinate-dependent process by preincubating the cells with NPA before adding atpenin A5. As shown in **Figure 8** panels C and F, the MitoSOX Red fluorescence intensity in the NPA-treated cells was markedly reduced indicating that the atpenin A5-induced MitoSOX Red oxidation is a succinate-dependent event. Collectively, these data indicate that atpenin A5 induces succinate-dependent O_2^- production from the mitochondrial complex II within intact DLD-1 and HT-29 cells. In agreement with the results from the isolated mitochondria, addition of atpenin A5 to the MitoSOX Red-loaded dermal fibroblasts did not display any red fluorescence (**Figure 8** panel G) indicating that atpenin A5 does not induce O_2^- production in mitochondria of dermal fibroblasts. But dermal fibroblasts showed an increment in red fluorescence with antimycin A (**Figure 8** panel H). This observation shows that the mitochondria of the dermal fibroblasts can produce O_2^- when the Q site of complex III is inhibited by antimycin A.

4. Discussion

ROS are produced from the mitochondrial complex II of the cancer cells (DLD-1 and HT-29) but not from the normal cells (dermal fibroblasts) when the entry of electrons to its quinone-binding site is blocked with 10 μM atpenin A5. However, previous studies in our laboratory have revealed that quinone reduction in the mitochondrial complex II can be completely inhibited at the tested concentration of atpenin A5 [8, 10]. Therefore, the difference in ROS production between cancer cells and normal cells may be attributed to a difference in the complex IIs that is upstream of the atpenin A5-binding site. Since the dicarboxylate-binding site inhibitor, NPA, could inhibit the ROS production induced by atpenin A5 in cancer cells, it is concluded that the ROS-producing site must be within the complex II, upstream of the atpenin A5 inhibition site(Q site) and downstream of NPA-binding site (dicarboxylate-binding site). According to previous evidence, the most likely ROS-producing sites of mitochondrial complex II are FAD [35–37] site and the Q site [37–39]. Observations of this study show that the ROS-generating site of complex II in atpenin A5-treated cancer cells is FAD but not Q site.

In order to explain this observation, we analyzed the published data on the crystal structures of flavoproteins, which are available in both the substrate bound and unbound forms [40–42]. They have shown that the capping domain in Fp subunit rotates during the enzyme catalysis. When the active site is not occupied by the substrate, the capping domain is rotated away from the active site creating a solvent channel linking the active site and the surrounding aqueous environment. In the substrate bound state, the capping domain is rotated inward concealing the active site, preventing the access of solvent to the active site [37]. Thus, closed conformation acquired during enzyme catalysis appears to be vital for

preventing the electron leak into oxygen. It appears that differences in the three-dimensional structure of the capping domain of the enzyme contribute to the difference in ROS production in cancer cells and normal cells. In fact, previous studies in our laboratory have shown that DLD-1 and HT-29 cells display FRD activity associated with the phosphorylation status of the Fp subunit of complex II and the degree of phosphorylation has been increased under hypoxic and hypoglycemic conditions [11]. If phosphorylation of amino acid residues in the Fp subunit can affect the correct movement of the capping domain during succinate oxidation, it will be possible for the electrons to leak to oxygen from the FAD site.

Our observation of a 1.5-fold higher ROS production from isolated mitochondria of DLD-1 cells than that of HT-29 cells may also have resulted due to the difference in the phosphorylation status of the two isoforms because the amino acids, which differ in the two isoforms, are found in the C-terminal domain of the Fp subunit, which plays an important role in opening and closing of the active site of the complex II enzymes [9] Substitution of tyrosine 629 in Fp I with phenylalanine in Fp II may affect the phosphorylation state of the C-terminal domain since tyrosine is a possible phosphorylation site while phenylalanine is not. This difference may affect the closing of the active site in Fp II during enzyme catalysis and allow more electrons to leak to oxygen from FAD. However, it is essential to resolve the crystal structure of the two isoforms of the human mitochondrial complex II with both the open and closed states of the capping domain and to establish the relationship between the phosphorylation status of the Fp subunit and the ROS production.

In summary, complex II Q site inhibitor atpenin A5 differentially affects the ROS production in normal cells and cancer cells. This difference may be utilized for selective destruction of cancer cells using complex II-inhibiting mitocans. We hypothesize that the difference in ROS production observed in this study is attributed to the difference in posttranslational modifications of mitochondrial complex II between normal cells and cancer cells, which may be more pronounced under hypoxic and hypoglycemic tumor microenvironments.

Acknowledgements

Ministry of Education, Culture and Sports in Japan is gratefully acknowledged for their financial support.

Author details

Madhavi P. Paranagama[1]* and Kiyoshi Kita[2]

*Address all correspondence to: madhaviparanagama@gmail.com

1 Department of Basic Sciences, Faculty of Dental Sciences, University of Peradeniya, Peradeniya, Sri Lanka

2 School of Tropical Medicine and Global Health, Nagasaki University, Nagasaki, Japan

References

[1] Alston CL, Rocha MC, Lax NZ, Turnbull DM, Taylor RW. The genetics and pathology of mitochondrial disease. The Journal of Pathology. 2017;**241**(2):236-250

[2] Hoekstra AS, Bayley JP. The role of complex II in disease. Biochimica et Biophysica Acta. 2013;**1827**(5):543-551

[3] Kita K, Shiomi K, Omura S. Advances in drug discovery and biochemical studies. Trends in Parasitology. 2007;**23**(5):223-229

[4] Sakai C, Tomitsuka E, Esumi H, Harada S, Kita K. Mitochondrial fumarate reductase as a target of chemotherapy: From parasites to cancer cells. Biochimica et Biophysica Acta. 2012;**1820**(5):643-651

[5] Kluckova K, Bezawork-Geleta A, Rohlena J, Dong L, Neuzil J. Mitochondrial complex II, a novel target for anti-cancer agents. Biochimica et Biophysica Acta. 2013;**1827**(5):552-564

[6] Ackrell BA. Progress in understanding structure-function relationships in respiratory chain complex II. FEBS Letters. 2000;**466**(1):1-5

[7] Iverson TM. Catalytic mechanisms of complex II enzymes: A structural perspective. Biochimica et Biophysica Acta. 2013;**1827**(5):648-657

[8] Tomitsuka E, Goto Y, Taniwaki M, Kita K. Direct evidence for expression of type II flavoprotein subunit in human complex II (succinate-ubiquinone reductase). Biochemical and Biophysical Research Communications. 2003;**311**(3):774-779

[9] Tomitsuka E, Hirawake H, Goto Y, Taniwaki M, Harada S, Kita K. Direct evidence for two distinct forms of the flavoprotein subunit of human mitochondrial complex II (succinate-ubiquinone reductase). Journal of Biochemistry. 2003;**134**(2):191-195

[10] Sakai C, Tomitsuka E, Miyagishi M, Harada S, Kita K. Type II Fp of human mitochondrial respiratory complex II and its role in adaptation to hypoxia and nutrition-deprived conditions. Mitochondrion. 2013;**13**(6):602-609

[11] Tomitsuka E, Kita K, Esumi H. Regulation of succinate-ubiquinone reductase and fumarate reductase activities in human complex II by phosphorylation of its flavoprotein subunit. Proceedings of the Japan Academy. Series B, Physical and Biological Sciences. 2009;**85**(7):258-265

[12] Cecchini G. Function and structure of complex II of the respiratory chain. Annual Review of Biochemistry. 2003;**72**:77-109

[13] Kita K, Takamiya S. Electron-transfer complexes in Ascaris mitochondria. Advances in Parasitology. 2002;**51**:95-131

[14] Hirayama A, Kami K, Sugimoto M, Sugawara M, Toki N, Onozuka H, et al. Quantitative metabolome profiling of colon and stomach cancer microenvironment by capillary electrophoresis time-of-flight mass spectrometry. Cancer Research. 2009;**69**(11):4918-4925

[15] Murphy MP. How mitochondria produce reactive oxygen species. The Biochemical Journal. 2009;**417**(1):1-13

[16] Jezek P, Hlavata L. Mitochondria in homeostasis of reactive oxygen species in cell, tissues, and organism. The International Journal of Biochemistry & Cell Biology. 2005;**37**(12):2478-2503

[17] Messner KR, Imlay JA. Mechanism of superoxide and hydrogen peroxide formation by fumarate reductase, succinate dehydrogenase, and aspartate oxidase. The Journal of Biological Chemistry. 2002;**277**(45):42563-42571

[18] Guo J, Lemire BD. The ubiquinone-binding site of the *Saccharomyces cerevisiae* succinate-ubiquinone oxidoreductase is a source of superoxide. The Journal of Biological Chemistry. 2003;**278**(48):47629-47635

[19] Ishii N, Fujii M, Hartman PS, Tsuda M, Yasuda K, Senoo-Matsuda N, et al. A mutation in succinate dehydrogenase cytochrome b causes oxidative stress and ageing in nematodes. Nature. 1998;**394**(6694):694-697

[20] Senoo-Matsuda N, Yasuda K, Tsuda M, Ohkubo T, Yoshimura S, Nakazawa H, et al. A defect in the cytochrome b large subunit in complex II causes both superoxide anion overproduction and abnormal energy metabolism in *Caenorhabditis elegans*. The Journal of Biological Chemistry. 2001;**276**(45):41553-41558

[21] Ishii T, Yasuda K, Akatsuka A, Hino O, Hartman PS, Ishii NA. Mutation in the SDHC gene of complex II increases oxidative stress, resulting in apoptosis and tumorigenesis. Cancer Research. 2005;**65**(1):203-209

[22] Huang J, Lemire BD. Mutations in the *C. elegans* succinate dehydrogenase iron-sulfur subunit promote superoxide generation and premature aging. Journal of Molecular Biology. 2009;**387**(3):559-569

[23] Zhao Z, Rothery RA, Weiner JH. Effects of site-directed mutations in *Escherichia coli* succinate dehydrogenase on the enzyme activity and production of superoxide radicals. Biochemistry and Cell Biology. 2006;**84**(6):1013-1021

[24] Szeto SS, Reinke SN, Sykes BD, Lemire BD. Ubiquinone-binding site mutations in the *Saccharomyces cerevisiae* succinate dehydrogenase generate superoxide and lead to the accumulation of succinate. The Journal of Biological Chemistry. 2007;**282**(37):27518-27526

[25] Guzy RD, Sharma B, Bell E, Chandel NS, Schumacker PT. Loss of the SdhB, but not the SdhA, subunit of complex II triggers reactive oxygen species-dependent hypoxia-inducible factor activation and tumorigenesis. Molecular and Cellular Biology. 2008;**28**(2):718-731

[26] Cervera AM, Apostolova N, Crespo FL, Mata M, McCreath KJ. Cells silenced for SDHB expression display characteristic features of the tumor phenotype. Cancer Research. 2008;**68**(11):4058-4067

[27] Dong LF, Low P, Dyason JC, Wang XF, Prochazka L, Witting PK, et al. Alpha-tocopheryl succinate induces apoptosis by targeting ubiquinone-binding sites in mitochondrial respiratory complex II. Oncogene. 2008;**27**(31):4324-4335

[28] Dong LF, Freeman R, Liu J, Zobalova R, Marin-Hernandez A, Stantic M, et al. Suppression of tumor growth in vivo by the mitocan alpha-tocopheryl succinate requires respiratory complex II. Clinical Cancer Research. 2009;**15**(5):1593-1600

[29] Angulo-Molina A, Reyes-Leyva J, Lopez-Malo A, Hernandez J. The role of alpha tocopheryl succinate (alpha-TOS) as a potential anticancer agent. Nutrition and Cancer. 2014;**66**(2):167-176

[30] Miyadera H, Shiomi K, Ui H, Yamaguchi Y, Masuma R, Tomoda H, et al. Atpenins, potent and specific inhibitors of mitochondrial complex II (succinate-ubiquinone oxidoreductase). Proceedings of the National Academy of Sciences of the United States of America. 2003;**100**(2):473-477

[31] Mohanty JG, Jaffe JS, Schulman ES, Raible DG. A highly sensitive fluorescent microassay of H_2O_2 release from activated human leukocytes using a dihydroxyphenoxazine derivative. Journal of Immunological Methods. 1997;**202**(2):133-141

[32] Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ. Production of reactive oxygen species by mitochondria: Central role of complex III. The Journal of Biological Chemistry. 2003;**278**(38):36027-36031

[33] Mukhopadhyay P, Rajesh M, Hasko G, Hawkins BJ, Madesh M, Pacher P. Simultaneous detection of apoptosis and mitochondrial superoxide production in live cells by flow cytometry and confocal microscopy. Nature Protocols. 2007;**2**(9):2295-2301

[34] Robinson KM, Janes MS, Beckman JS. The selective detection of mitochondrial superoxide by live cell imaging. Nature Protocols. 2008;**3**(6):941-947

[35] Ohnishi T, King TE, Salerno JC, Blum H, Bowyer JR, Maida T. Thermodynamic and electron paramagnetic resonance characterization of flavin in succinate dehydrogenase. The Journal of Biological Chemistry. 1981;**256**(11):5577-5582

[36] Maklashina E, Iverson TM, Sher Y, Kotlyar V, Andrell J, Mirza O, et al. Fumarate reductase and succinate oxidase activity of *Escherichia coli* complex II homologs are perturbed differently by mutation of the flavin binding domain. The Journal of Biological Chemistry. 2006;**281**(16):11357-11365

[37] Paranagama MP, Sakamoto K, Amino H, Awano M, Miyoshi H, Kita K. Contribution of the FAD and quinone binding sites to the production of reactive oxygen species from *Ascaris suum* mitochondrial complex II. Mitochondrion. 2010;**10**(2):158-165

[38] Salerno JC, Ohnishi T. Studies on the stabilized ubisemiquinone species in the succinate-cytochrome c reductase segment of the intact mitochondrial membrane system. The Biochemical Journal. 1980;**192**(3):769-781

[39] Tran QM, Rothery RA, Maklashina E, Cecchini G, Weiner JH. The quinone binding site in *Escherichia coli* succinate dehydrogenase is required for electron transfer to the heme b. The Journal of Biological Chemistry. 2006;**281**(43):32310-32317

[40] Yamashita T, Ino T, Miyoshi H, Sakamoto K, Osanai A, Nakamaru-Ogiso E, et al. Rhodoquinone reaction site of mitochondrial complex I, in parasitic helminth, *Ascaris suum*. Biochimica et Biophysica Acta. 2004;**1608**(2-3):97-103

[41] Yankovskaya V, Horsefield R, Tornroth S, Luna-Chavez C, Miyoshi H, Leger C, et al. Architecture of succinate dehydrogenase and reactive oxygen species generation. Science. 2003;**299**(5607):700-704

[42] Yu L, Xu JX, Haley PE, Yu CA. Properties of bovine heart mitochondrial cytochrome b560. The Journal of Biological Chemistry. 1987;**262**(3):1137-1143