論文の内容の要旨

論文題目　Reactive oxygen species (ROS) production from the mitochondrial complex II (succinate-ubiquinone oxidoreductase)

和訳　呼吸鎖複合体Ⅱ(コハク酸-ユビキノン酸化還元酵素)による活性酸素種(ROS)産生の解析

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Human mitochondrial complex II functions as a succinate-ubiquinone oxidoreductase (SQR) coupling the succinate oxidase activity in the TCA cycle to reduction of ubiquinone in the respiratory chain. It is unique among the TCA cycle enzymes as the only membrane bound enzyme and among the respiratory chain enzymes as the only enzyme that is exclusively encoded by nuclear genes (Cecchini, 2003). Recently, human mitochondrial complex II is identified as a tumour suppressor (Gottlieb and Tomilson, 2005). Generally, proper expression of tumour suppressor proteins is essential to prevent carcinogenesis. Down regulated expression or expression of a mutated form of a tumour suppressor facilitates oncogenesis. In accordance with this phenomenon, expression of mutated iron sulphur (Ip), cytochrome *b* large (C*yb*L) or cytochrome *b* small (C*yb*S) subunits in human mitochondrial complex II has been shown to cause several types of hereditary as well as sporadic tumours, primarily the pheochromocytoma (in chromaffin cells of the adrenal medulla) and paraganglioma (in sympathetic and parasympathetic ganglia). (Baysal et al., 2000;

Srirangalingam et al., 2007; Timmers et al., 2009). In addition, breast, thyroid and renal carcinomas are found to be associated with mutations in complex II (Neumann et al., 2004; Ni et al., 2008). Despite extensive genetic evidence highlighting the importance of complex II mutations in carcinogenesis, the biochemical mechanism linking complex II mutations to carcinogenesis is not elucidated yet. Among the several proposals emerged to explain this link, one of the leading proposals is that tumour formation might be associated with over production of reactive oxygen species (ROS) from mutated complex II (Astuti et al., 2001; Rustin and Rotig, 2002). Hence, understanding the mechanism of ROS production from human mitochondrial complex II is important for effective clinical interventions of complex II associated cancers.

Most of the studies related to understanding the ROS production from human mitochondrial complex II have been conducted on model organisms such as *Escherichia coli, Saccharomyces cerevisiae and Caenorhabditis elegans*. A hallmark in these studies is the finding that the *mev-1* mutation in the mitochondrial complex II of *C. elegans* results in enhanced superoxide production and oxygen hypersensitivity. 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 superoxide overproduction from mitochondria that leads to apoptosis and tumorigenesis. More recently, mutations in the Ip subunit in the *C. elegans* also has been found to display enhanced ROS production. Site directed mutagenesis of CybS subunit in *E. coli* and the Ip and CybS subunits in *S. cerevisea* also has shown enhanced ROS production providing further support for the hypothesis that reactive oxygen species underlies the complex II mutations associated carcinogenesis.

Indeed, some studies have been conducted on human cell lines using pharmacological and genetic interventions to inhibit complex II activity, but the results are controversial. To the best of our knowledge, ROS production from human mitochondrial complex II has been shown only by Guzy et al. (2008), in intact cells of the hepatoma cell line Hep3B by inhibiting the complex II activity by 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. (2008), failed to detect ROS production. However, both groups have found HIF-1 α stabilization when complex II activity is inhibited. In addition, Selak et al. (2005) and (2006) also have observed HIF-1 α stabilization when complex II activity was inhibited by RNAi of its CybS subunit in human embryonic kidney cell line (HEK293). Similar finding has been reported for Fp RNAi primary cardiomyocytes from rat heart. However, in these two instances, the HIF-1 α stabilization has been unrelated to ROS production. Whether the controversy in these results are due to the difference in the complex II subunit that is interfered, difference in the cell type or the methodological differences in ROS detection used by different research groups are yet to be answered.

Since the complex II in the anerobic respiratory chain couples the oxidation of quinol to reduction of fumarate, it is called as quinol fumarate reductase (QFR). Mitochondrial complex II in anaerobic organisms function in the reverse direction of SQR and couples the oxidation of quinol to reduction of fumarate (quinol fumarate reductase:QFR). These enzymes have the ability to catalyze the fumarate reductase (FRD) activity when the water-soluble dye methyl violegen is used as an artificial electron donor. Several lines of evidence indicate that mammalian mitochondrial complex II which classically functions as a SQR also can function in the reverse direction and catalyze FRD activity. The mitochondrial complex II in parasitic organisms such as *A. suum* exhibits well defined FRD activity (Kita and Takamiya, 2002). Recently, several research groups including my group have speculated that ROS production from the mitochondrial complex II is associated with fumarate reductase (FRD).

To test this hypothesis, first I used the parasitic nematode *Ascaris suum* as a model organism since it has well defined FRD activity in the mitochondrial complex II. Besides, the amino acid residues in the catalytic domains in mammalian mitochondrial complex II are well conserved in this enzyme. In *A. suum*, the adult worms and L3 larvae express two distinct isoforms of complex II. They differ structurally in the Fp and the CybS subunits. Functionally, the adult complex II serves as the terminal oxidase of the anaerobic respiratory chain and catalyzes FRD activity. In

addition, it shows a high SQR activity *in vitro.* The larval complex II is a component of the aerobic respiratory chain and the TCA cycle and functions as a SQR. However, it shows significant FRD activity also under *in vitro* conditions (Amino et al., 2003).

Mitochondria were isolated from the muscles of the adult and L3 larval stages, inside-out submitochondrial particles were prepared and washed to remove the antioxidant molecules that interfere with the accurate estimation of ROS. Superoxide and hydrogen peroxide production in the submitochondrial particles were determined by superoxide dismutase inhibitable acetylated cytochrome *c* reduction and the catalase inhibitable amplex red oxidation, respectively. Initially, ROS production from the entire respiratory chain was measured using either succinate or NADH as the respiratory substrate. Interestingly, a substantial amount of both superoxide and hydrogen peroxide production were detected from the adult respiratory chain while only superoxide could be detected from that of L3 larvae. The succinate-dependent superoxide production rate observed in the adult respiratory chain was approximately hundred fold higher than that from the L3 larvae. Subsequently, a series of respiratory chain inhibitors in combination with the respiratory substrates were employed in the assays in order to localize the site of ROS production. In *A. suum* adult worms, quinazoline (Q site inhibitor of complex I), atpenin A5 (Q site inhibitor of complex II) and malonate (dicarboxylate binding site inhibitor of complex II) were the inhibitors used. In L3 larvae, rotenone (Q site inhibitor of complex I), antimycin A (Qi site inhibitor of complex III), stigmatellin (Qo site inhibitor of complex III) and NaN_3 (complex IV inhibitor) were used together with the complex II inhibitors used in adults worms. When succinate was used as the respiratory substrate, addition of quinazoline (the Q site inhibitor of complex I) did not show a significant effect on the ROS production from the adult SMP. This implies that the contribution of the complex I for ROS production is not significant. Similarly, when NADH was used as the respiratory substrate, addition of either quinazoline(Q site inhibitor of complex I) or atpenin A5 (Q site inhibitor of complex II) resulted in a 95% suppression of the ROS production, indicating that contribution of the complex I for ROS production in the adult SMP is during NADH oxidation also is negligible. In L3 larvae,

when succinate was used as the substrate, addition of neither the complex I inhibitors nor the complex III inhibitors showed any significant effect on ROS production but, addition of atpenin A5 (Q site inhibitor of complex II) resulted in a marked reduction in the ROS production. Thus, when succinate is used as the substrate complex II is the source of ROS in L3 larvae SMP also. On the other hand, when NADH was used as the substrate, addition of the complex I, III or IV inhibitors increased the ROS production but addition of atpenin A5 did not show a significant effect. This implies that complex II in L3 larvae does not contribute for ROS production during NADH oxidation. Taken together, these data implicate that complex II is the major source of ROS in the *A. suum* respiratory chain when succinate is oxidized under aerobic conditions. Next, I attempted to localize the redox centres within complex II contributing for ROS production. In *E. coli*, the flavin of complex IIs is considered to be the source of ROS (Messner and Imlay, 2002) because at higher succinate concentrations (around 50-100 mM), it is completely inhibited. Excess succinate is suggested to suppress ROS production by hindering the access of oxygen to FAD, thus preventing its autoxidation. When I measured the succinate-dependent ROS production in *A. suum* SMP, it increased with substrate concentration and was highest at 0.5-1 mM succinate in both adults and L_3 larvae and gradually decreased along with increasing succinate concentrations. However, the percentage of inhibition in ROS production at ≥100 mM succinate in *A. suum* complex II was only 80%, with 20% always remaining. Such residual ROS production observed even after complete blocking of the FAD site with succinate suggests another site for ROS production, in addition to FAD. The Q site is the other candidate for a potential source of ROS in complex II. To determine the contribution of the Q site of *A. suum* complex II to ROS production, I analyzed its ROS production in the presence of atpenin A5, which inhibits the Q site of complex II completely. $O_2^{\cdot-}$ production from complex II of *A. suum* adult worms and L3 larvae with 0.75 mM succinate was decreased 38% and 14% respectively, in the presence of atpenin A5. Thus, it appears that electrons leak to oxygen from the Q site also during succinate oxidation. Atpenin A5 may inhibit either binding and/or reduction of quinone, thus preventing electron flow in the Q site and inhibiting ROS

production. Overall, the FAD site contributed for the major fraction of the ROS production with a lower contribution from Q site when succinate is oxidized by *A. suum* complex II under aerobic conditions.

Motivated by finding a substantial ROS production from the mitochondrial complex II of *A. suum* which possesses well defined FRD activity, next I examined whether human mitochondrial complex II with FRD activity also have the potential to produce ROS. To test this possibility, ROS production was analyzed in the two human colon cancer cells lines HT-29 and DLD-1, which have been reported to exhibit significantly higher FRD activity than those from the normal cells (Tomitsuka et al., 2009). These two cell lines displayed predominant expression of type I and type II isoforms of the human complex II which differ in their Fp subunit. Human dermal fibroblasts served as the control from a non cancerous tissue with a mitochondrial complex II with low FRD activity. ROS production was measured in isolated mitochondria using amplex red assay in the presence of a cell permeable SOD. In this assay, superoxide molecules produced within mitochondria are converted to H_2O_2 by the cell permeable SOD and the H_2O2 diffusing out of the mitochondria are measured. To mimic the complex II mutations preventing the entry of electrons to Q site, atpenin A5, a potent and specific Q site inhibitor was used. According to the results, addition of atpenin A5 increased the H_2O_2 production that is totally inhibited by nitropropionic acid, a succinate analogue that inhibits succinate oxidation irreversibly in complex II.

To find whether the ROS production detected in the isolated mitochondria is applicable to the live cells also, superoxide production in HT-29 and DLD-1 cells grown into a monolayer on glass cover slips was detected in the presence and absence of atpenin A5 using the superoxide sensitive probe mitosox red. The confocal images of the cells treated with atpenin A5 showed a clear red flouresecense around the nucleus that colocalized with the mitochondrial specific dye mitotracker green. Pretreatment of the cells with SOD or nitropropionic acid before the addition of atpenin A5 prevented the development of mitosox fluorescence indicating that increase in the mitosox fluorescence induced by atpenin A5 is dependent on superoxide originating from complex

II. Untreated cells or cells treated with the vehicle (0.4% DMSO) did not show a detectable fluorescence in mitosox red. 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. It implies that inhibition of the Q site of complex II in dermal fibroblasts does not lead to ROS production from redox centres proximal to Q site.

In 2008, it has been reported that ROS originating specifically from mitochondrial complex II can induce normoxic HIF-1 stabilization. In the present study, HIF-1 stabilization was used to confirm the atpenin A5 induced ROS production from mitochondrial complex II in human cells that could be detected with amplex red and mitosox assays. Western blotting for whole cell extracts from HT-29 and DLD-1 cells treated with atpenin A5 showed an increase in the steady state level of HIF-1 α protein. It was markedly reduced or completely disappeared when the cells were pretreated with the ROS scavenger, N acetyl cysteine. HIF-1 α was not detected when the cells were pretreated with nitropropionic acid. Overall, the data of the western blots demonstrates that succinate-dependant ROS production from mitochondrial complex II leads to an increase in the steady state level of HIF-1 α in the HT-29 and DLD-1 cells.

When results of the ROS production from human cancer cells with FRD activity are collectively considered, it appears that that the inhibition of the entry of electron derived from succinate oxidation into the Q site in the complex II of cancer cells results in an aberrant leakage of electrons to oxygen. This finding indicates that either the FAD site and/or the Fe-S clusters are contributing for ROS production in cancer cell complex II. In this regard, the crystallographic studies on mitochondrial complex II have demonstrated that FAD site is localized superficially while the Fe-S clusters are embedded with the protein molecule. Besides, a flavin radical with the potential to leak electrons to oxygen is known to form at the FAD site during the normal electron transfer process through complex II. Therefore, FAD site is the most likely ROS producing site when the Q site is blocked.

It has been known that the FRD activity in the mitochondrial complex II in the DLD-1 and HT-29 cells is connected with the phosphorylation status of the Fp subunit of the enzyme (Tomitsuka et al., 2009). Phosphorylation of a protein may affect its three dimensional structure. One possible explanation for the production of ROS from the complex II in the cancer cells but not from the normal cells may lie in the difference between the three dimensional structure of the capping domain of the Fp subunit that is imparted by the difference in the level of phosphorylation. It has been found 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 inwards concealing the active site, preventing the access of solvent to the active site (Lancaster et al., 2001). Thus, closed confirmation acquired during enzyme catalysis appears to be vital for preventing the electron leak into oxygen. For instance, if the 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. To test this hypothesis, it is essential to resolve the crystal structure of human 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 sub unit and the ROS production.