ALA-Porphyrin Science

The Effect of 5-Aminolevulinic Acid on Cytochrome *c* Oxidase Activity

Yuta Sugiyama,[a] Yuichiro Hagiya,[a] Tetsuya Hayashi,[b] Kyoko Tsuchiya,[c] Masahiro Ishizuka,[c] Tohru Tanaka,[c] Ichiro Okura[a] and Shun-ichiro Ogura[a]

[a] *Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259-B102, Nagatsuta-cho, Midori-ku, Yokohama, 226-8501, Japan. Fax: +81-45-924-5845; Tel: +81-45-924-5845; E-mail: sogura@bio.titech.ac.jp*

[b] *Third Department of Medicine, Osaka Medical College, 2-7 Daigakumachi, Takatsuki, Osaka 569-8686, Japan.*

[c] *SBI ALA PROMO CO., LTD., Izumi Garden Tower 20F, 1-6-1, Roppongi, Minato-ku, Tokyo,106-6020, Japan.*

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Summary

Cytochrome *c* oxidase (COX, Complex IV) is a heme enzyme in the electron transport chain of mitochondria. In the case of tumor or lifestyle disease, the decreased activity of COX is reportedly observed, indicating the importance of rescuing COX inactivation. In this study, we administered 5-aminolevulinic acid (ALA), the precursor in the porphyrin synthesis pathway leads to heme, to mice for 10 consecutive days. As a result, the markedly increase of COX protein levels and activity were observed in liver after ALA injection. In conclusion, our study suggests that administration of ALA promotes aerobic energy metabolism.

Keyword:

5-Aminolevulinic acid; Cytochrome *c* oxidase; Heme; Electron transport chain

Introduction

The main ATP synthesis pathway of aerobic organism is oxidative phosphorylation. During oxidative phosphorylation, electrons are transferred from electron donors to electron acceptors such as oxygen, in redox reactions. These redox reactions generate energy, which is used to convert ADP to ATP. These redox reactions are carried out by a series of protein complexes within mitochondrial inner membrane. These proteins are called electron transport chain. There are five main protein complexes. In this study, we focused on the last complex of electron transport chain (complex IV).

Complex IV, called cytochrome *c* oxidase (COX), is a key enzyme at the end of the respiratory electron transport chain. COX is a hemoprotein containing hemes as active site and performs an oxidation reaction of cytochrome *c* and a deoxidation reaction at the same time. Defects in mitochondrial function are associated with numerous neurodegenerative diseases (Parkinson's, Alzheimer's and Huntington's disease) and in particular with mitochondrial diseases. Mitochondrial diseases are characterized by defects in the oxidative phosphorylation system. Especially it is known that COX activity decline due to a lot of disease or aging [1, 2]. Therefore, it is important to rescue COX inactivation.

In most animals, heme is synthesized from glycine and succinyl-CoA from TCA cycle in a complex pathway involving eight enzymes. The first and normally rate-controlling step is carried out by the mitochondrial enzyme 5-aminolevulinic acid synthase (ALAS). Thus we consider that

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ALA would be imported into cytoplasm and result in upregulating heme biosynthesis by addition of ALA. Then, this heme-upregulation would make some effects on hemoproteins (Figure 1).

In this study, we make a hypothesis that addition of ALA would upregulate heme biosynthesis, and result in upregulation of COX protein. Therefore, we attempt to examine the effect of ALA-treatment on COX.

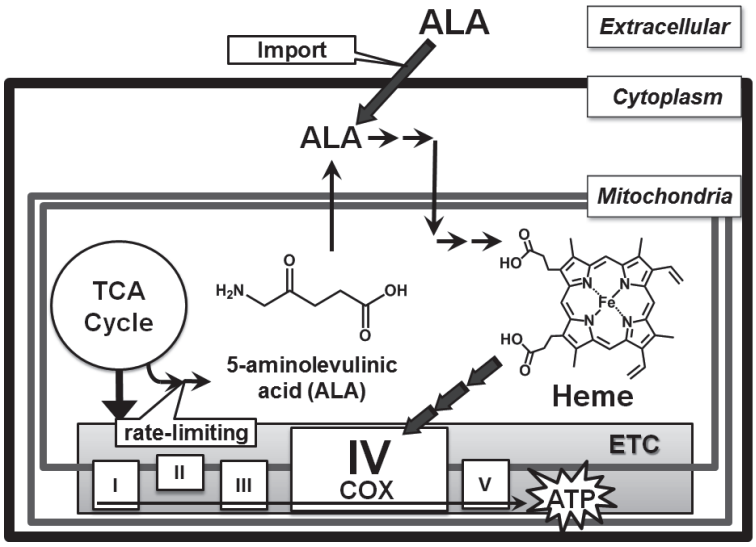

Figure 1. Schematic Illustration of Heme Biosynthesis
Lines are indicate cellular membrane (black) and mitochondrial membrane (gray). Heme biosynthesis pathway is initiated by the synthesis of ALA from glycine and succinyl-CoA from TCA cycle. The rate-limiting enzyme responsible for this reaction is ALA synthase. Therefore addition of ALA would result in upregulating heme biosynthesis and make some effects on hemoproteins.

Experimentals

Cell line and *in vitro* experiments

The UV♀2 (mouse vascular endothelial cell line) was used in this study. The cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (FBS) and ABAM, at 37 °C in an incubator with a controlled humidified atmosphere containing 5% CO_2. Where indicated, ALA was added to medium at a final concentration of 1 mM.

Animals and *in vivo* experiments

C57BL/6N male mice at 8 weeks of age were used in this study. Animals were injected ALA solution dissolved in saline or vehicle (saline) 100 mg/kg/day i.p. for 10 consecutive days. At the end of this period, the mitochondrial fractions were extracted from these mice tissues and used for measurement of COX activity and western blot analysis.

Measurement of COX activity

Measurement of COX activity was performed as previously described with some modifications [3]. Mouse tissues were extracted, and mitochondrial fractions were obtained using a Mitochondria Isolation Kit (MITOISO1, Sigma-Aldrich). In brief, fresh tissues were washed and homogenized (rotor-stator homogenizer (T10 basic Ultra-Turrax®, IKA, Staufen, Germany) at 15,000 rpm) in extraction reagent. The homogenate was centrifuged at 600 ×*g* for 5 min, and then the supernatant was further centrifuged at 11,000 ×*g* for 10 min. The pellet was suspended in storage buffer and used as a mitochondrial fraction. Protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories, CA). COX activity was measured using a Cytochrome *c* Oxidase Assay Kit (Sigma-Aldrich). In brief, 2.5 μg of the mitochondrial fraction were diluted with

enzyme dilution buffer containing 1 mM n-dodecyl β-d-maltoside. Ferrocytochrome *c* (reduced cytochrome *c* with dithiothreitol) was then added to the sample, and COX activity was measured by the decrease in absorption at 550 nm. The difference in extinction coefficients between reduced and oxidized cytochrome *c* is 21.84 at 550 nm [4]. One unit of COX activity was defined as the oxidization of 1.0 μmole of ferrocytochrome *c* per min at pH 7.0 at 25 °C.

Quantitative real-time PCR

Total RNA was prepared from 0.5×10^6 cells using a High Pure RNA Isolation Kit (Roche). RNA (1□μg) was added as a template to reverse-transcriptase reactions carried out using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCRs were set up using a SYBR® Premix Ex Taq™ (TaKaRa) and were cycled according to TaKaRa's instructions for Thermal Cycle Dice Real Time System. Experimental Ct values were normalized to glyceraldehyde-3-phosphate dehydrogenase (Gapdh), and relative mRNA expression was calculated versus a reference sample.

Western blot analysis

Western blot analysis was performed as previously described with some modifications [3]. Liver mitochondria, obtained as described above, were separated by 15% SDS-PAGE and then transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA). After incubating in 5% nonfat dry milk in TBST (25 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) at room temperature for 1 h, the membranes were incubated for 1 h with mouse anti-COX IV-1 antibody (1:200 dilution in TBST with 5% nonfat dry milk, Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation in a secondary anti-mouse IgG coupled to horseradish peroxidase (1:3000 dilution in TBST, Cell Signaling, Danvers, MA). Target proteins were detected using an ECL kit (GE Healthcare, UK), and the image was captured using an LAS-4000 imager (GE Healthcare).

Results and discussion

The effect of ALA on COX *in vitro*

We have first tested whether COX expressions are change by addition of ALA *in vitro*. COX is a membrane protein composed of 13 different subunits encoded by nuclear and mitochondrial genes in mammals [5]. In mammals, three of the subunits are encoded by mitochondrial genes and the ten other subunits are encoded by the unlinked nuclear genes. The largest nuclear encoded subunit, COX IV, due to the location and contact sites with the catalytic subunits 1 and 2, appears an attractive candidate for the regulation of COX activity [6]. In this study, we focus on the COX IV subunit 1 as representative.

UV♀2 cells were incubated in medium containing 1 mM of ALA for 4 hours, the quantity of COX IV protein and Cox 4 mRNA levels were measured (Figure 2A and 2B), showing both of COX levels were strongly elevated. These results indicate that incubation with ALA induces COX expression.

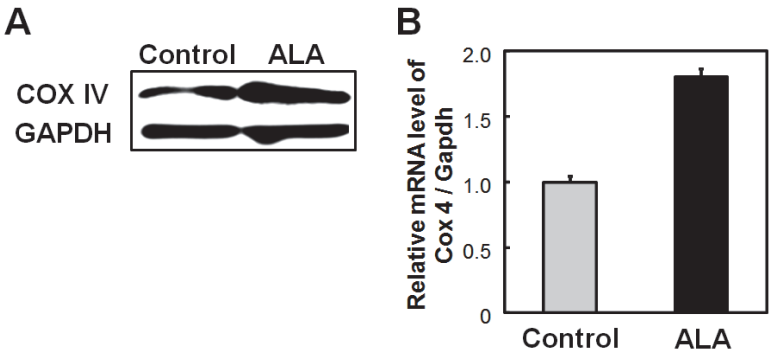

Figure 2. COX protein and mRNA levels
UV♀2 cells (mouse vascular endothelial cell line) were incubated for 4 hour with ALA at a final concentration of 1 mM (ALA) or vehicle (control). COX IV protein expression was measured by western blot analysis (A). Cox 4 mRNA levels were measured by real time PCR (B).

The effect of ALA on COX *in vivo*

In order to explore the effect of ALA on COX activity *in vivo*, we administered ALA dissolved in saline and give 100 mg/kg/day i.p. to C57BL/6N mice at 8 weeks of age. Using mitochondrial fractions of each tissue extracted from ALA administered mice, COX enzyme activity was measured by the decrease in absorption at 550 nm of ferrocytochrome *c*.

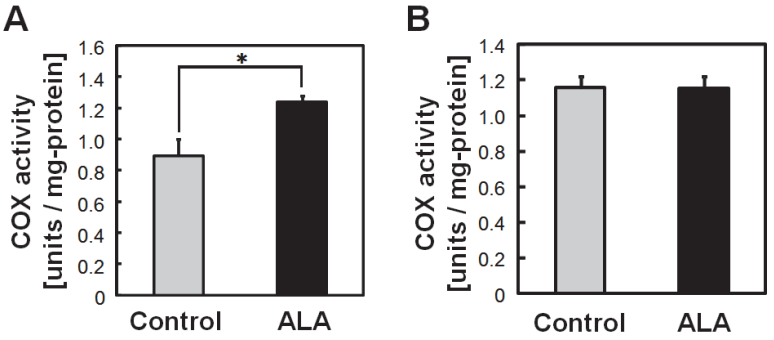

Figure 3. ALA induced activation of COX
COX activity was measured in liver (A, *; $p < 0.05$) or brain (B). Determination of COX activity is based on the observation of a decrease in absorbance at 550 nm of ferrocytochrome *c* oxidized by COX.

COX activity was significantly increase by 40% in liver from ALA administered mice compared with control group ($p < 0.05$; Figure 3A). This result clearly demonstrates that ALA administration led to a significant upregulation of COX activity in liver. On the other hand, same effect was not observed in the case of brain (Figure 3B). The organs mainly involved in heme biosynthesis are liver and bone marrow. Therefore conceivably, these data indicate that ALA inducible effect occurs mainly in liver.

Expression levels of COX protein were measured by western blot analysis. Figure 4 shows COX protein levels in liver. By Ponceau S staining of PVDF membrane, it was confirmed that same protein volumes of liver mitochondrial fractions were used for SDS-polyacrylamide gel electrophoresis (Figure 4A). The levels of COX IV were increased by 40% in ALA-treated animals (Figure 4B and 4C). These data indicate that ALA administration causes up-regulation of COX protein level.

Many important hemoproteins, the superfamily of proteins containing a heme cofactor such as cytochrome P450 and catalase, are particularly high concentrations occurring in the liver. Therefore, although organ specificity, effects of ALA administration are very meaningful.

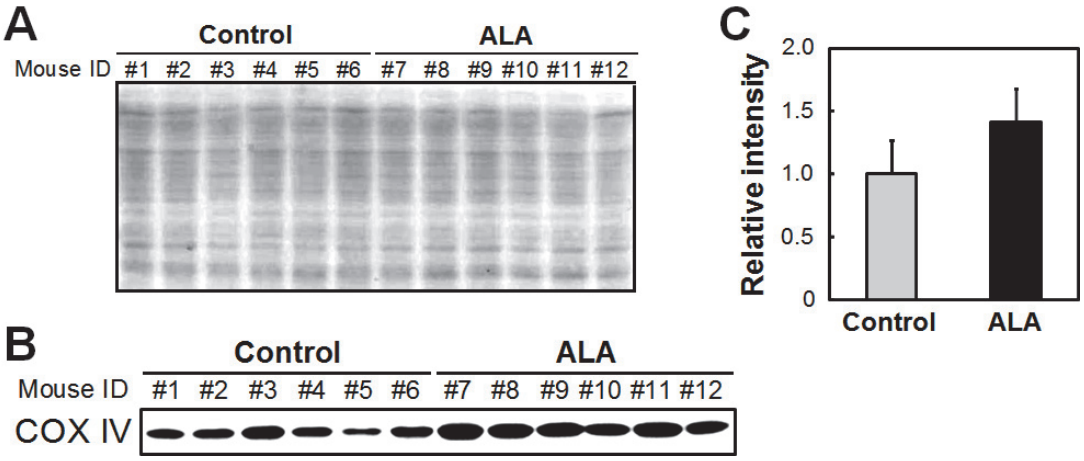

Fig. 4 ALA up-regulated protein levels of COX IV
COX protein levels of the mouse liver mitochondrial fraction were examined by western blot analysis. Transferred proteins were visualized by Ponceau S staining (A). COX IV proteins were detected by western blot analysis (B) and quantified (C).

Conclusion

Our experiments *in vitro* revealed that ALA-treated cells were enhanced synthesis of COX (Figure 2). In the ALA-administered mouse livers, both COX protein expression and activity were increased (Figure 3 and 4). These data indicate that ALA administration increases COX protein levels, and results in up-regulate of COX activity.

In conclusion, our study suggests that administration of ALA promotes aerobic energy metabolism. Consequently, ALA can induce progress in healthcare field. Especially, ALA treatment could be an answer to some diseases by rescuing of mitochondrial function, such as diabetes, Alzheimer and lifestyle disease. As future study, we would like to research the effect of ALA on other hemoproteins.

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