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Mitochondrial LON Protease-Dependent Degradation of Cytochrome c Oxidase Subunits under Hypoxia and Myocardial Ischemia

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Abstract

The mitochondrial ATP dependent matrix protease, Lon, is involved in the maintenance of mitochondrial DNA nucleoids and degradation of abnormal or misfolded proteins. The Lon protease regulates mitochondrial Tfam (mitochondrial transcription factor A) level and thus modulates mitochondrial DNA (mtDNA) content. We have previously shown that hypoxic stress induces the PKA-dependent phosphorylation of cytochrome c oxidase (CcO) subunits I, IVi1, and Vb and a time-dependent reduction of these subunits in RAW 264.7 murine macrophages subjected to hypoxia and rabbit hearts subjected to ischemia/reperfusion. Here, we show that Lon is involved in the preferential turnover of phosphorylated CcO subunits under hypoxic/ischemic stress. Induction of Lon protease occurs at six to twelve hours of hypoxia and this increase coincides with lower CcO subunit contents. Over-expression of flag-tagged wild type and phosphorylation site mutant Vb and IVi1 subunits (S40A and T52A, respectively) caused marked degradation of wild type protein under hypoxia while the mutant proteins were relatively resistant. Furthermore, the recombinant purified Lon protease degraded the phosphorylated IVi1 and Vb subunits, while the phosphorylation-site mutant proteins were resistant to degradation. 3D structural modeling shows that the phosphorylation sites are exposed to the matrix compartment, accessible to matrix PKA and Lon protease. Hypoxic stress did not alter CcO subunit levels in Lon depleted cells, confirming its role in CcO turnover. Our results therefore suggest that Lon preferentially degrades the phosphorylated subunits of CcO and plays a role in the regulation of CcO activity in hypoxia and ischemia/reperfusion injury.

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Mitochondrial LON; CcO subunits; PKA dependent phosphorylation; Hypoxia; Heart Ischemia; 3D modeling

Introduction

Cytochrome c oxidase (CcO) is a multi-subunit terminal oxidase of mitochondrial electron transport chain complex (ETC) which plays a key role in the regulation of ATP production (1–4). The CcO is a highly regulated enzyme whose activity is modulated by O_2 tension, membrane lipid environment, hormonal regulation and also by protein modification (1;2;4;5). Altogether, 18 putative phosphorylation sites have been identified on the 13 subunits of this bigenomic complex under different physiological and pathological conditions (6). Lee et al. (7) showed that T304 phosphorylation of subunit I by a Tyr kinase under normal physiological conditions inhibits CcO activity. Work from our laboratory showed that subunits I, IVi1 and Vb of CcO complex (4;8;9) are reduced during hypoxia and myocardial ischemia/reperfusion stress in rodents and rabbits and these subunits are phosphorylated by a novel cAMP-independent mitochondrial matrix PKA (4). Using nanoLC MS-MS analysis, the myocardial ischemia/reperfusion-induced phosphorylation sites of CcO subunits were mapped to positions S115 and S116 of subunit I, S40 of mature subunit Vb and T52 of mature subunit IVi1 (10). Manfredi's group, on the other hand, showed that phosphorylation of subunit IVi1 by a mitochondrial cAMP-dependent PKA at Ser58 modulated an increase in CcO activity under physiological conditions possibly through modulation of ADP/ATP binding (11). Thus, reports from various groups suggest both inhibition and activation of CcO activity by site- specific phosphorylation of different subunits (12;13).

Altered CcO function is an important biomarker for a number of pathophysiological conditions (12;14–16). As we and others have shown, CcO activity is disrupted under myocardial ischemia/reperfusion injury due to selective reduction of some key subunits involved in the assembly of the complex or its stability (4;9;17–19). Reduced subunit levels have been shown in solid tumors (20) although a recent study showed that highly proliferative drug resistant tumor cells contain a robust CcO activity (21). Furthermore, reduced CcO activity is an important marker for a number of neurodegenerative diseases such as Parkinson's disease (22;23). Studies from our and other laboratories also imply that CcO dysfunction may directly or indirectly contribute to increased ROS production (4;24–26). More important, CcO disruption causes dissociation of ETC super-complexes consisting of Complex I, Complex III and complex IV which may be the source of ROS through electron spillage and also reduced respiration coupled ATP synthesis. It is therefore important to understand the precise mechanism of CcO subunit loss and loss of enzyme activity under hypoxia and ischemia/reperfusion injury.

In this paper we show that mitochondrial Lon protease, which is induced under hypoxia and ischemia/reperfusion plays a direct role in the degradation of nuclear encoded CcO subunits IVi1 and Vb. Importantly, PKA mediated phosphorylation functions as a signal for Lon-

mediated degradation of these subunits. Phosphorylation resistant mutant proteins S40A Vb and T52A IVi1 showed resistance to Lon mediated degradation in *in vitro* reactions reconstituted with purified Lon and also in cells exposed to hypoxia. Additionally, cells expressing T52A mutant IVi1 showed resistance to hypoxia-mediated loss of subunits as well as enzyme activity. These results for the first time demonstrate a mechanism for Lon mediated degradation of CcO under pathophysiological conditions.

Materials and Methods

Cell Types, Culture conditions and Hypoxia

RAW 264.7 mouse monocyte macrophages were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum as described before (10). Cells at 80–90% confluency were grown under hypoxic conditions (0.2% O_2) or normoxic conditions (21% O_2) for 6–12h as described before (10).

In our previous studies we used myocardial ischemia and hypoxia in RAW264.7 cells because they are highly sensitive to hypoxia (4;26;27). We also used C2C12 skeletal myocytes which are relatively more resistant to hypoxia. Furthermore most of our CcO knockdown studies were carried out with these cells. CcO KD by stable expression of shRNA induced extensive cell death. Furthermore, knock down of mitochondrial Lon protease mRNA was generated by our collaborator (42) in HCT116 colon cancer cells. We used these cells for testing the effects of Lon in CcO subunit degradation.

Ischemia and Perfusion Protocol

All animal procedures were carried out in conformance with the Guidelines for the Care and Use of Laboratory Animals from the National Institutes of Health. Hearts from New Zealand male white rabbits (2–2.5 kg) were removed after euthanasia and perfused using a Langendorff perfusion apparatus as described before (4;28). The aorta was cannulated and the heart suspended on a Langendorff perfusion apparatus in which the coronary arteries were perfused from a reservoir with oxygenated temperature-controlled Tyrode’s solution at a constant pressure of 80 mmHg. The heart was surrounded by a temperature – controlled 50 ml chamber with an overflow so that the heart was submerged in its effluence. Coronary flow was measured by a timed drip count from the overflow. Bipolar electrocardiograms (X and Y leads) were recorded by two pairs of 1cm diameter gold plated electrodes that were affixed to the walls of the chamber. In order to monitor contractility, a water filled latex balloon, connected to a pressure gauge was inserted into the left ventricular cavity. Following normal perfusion at about 37–40 ml/min for 20 min, Global ischemia was induced without complete disruption of coronary circulation by reducing aortic perfusion to 2.5ml/min for 20 min as described before (4). Control hearts were perfused at normal flow rates for 20 min. Following global ischemia, the hearts were perfused at normal rates for 1–2h to inflict reperfusion injury.

Isolation of mitochondria and assay of CcO activity

Mitochondria were isolated from cells and myocardial tissue by differential centrifugation of cell and tissue homogenates in mitochondria isolation buffer (70 mM sucrose, 220 mM

mannitol, 10 mM HEPES, pH 7.4, and 2 mM EDTA) as described before (29). CcO activity with isolated mitochondrial preparations was measured in cholate containing buffer at 550 nm in a final volume of 1 ml using a Cary-1E spectrophotometer (Varian Instruments, Walnut Creek, CA). The assay medium consisted of 100 μM reduced cytochrome c, 10 mM sodium phosphate buffer, pH 7.2 and 0.02% cholate. The decrease in absorbance at 550nm reflects the rate of oxidation of reduced cytochrome C by CcO (8). The CcO activity expressed as nMol cytochrome c oxidized/mg protein/min varied nearly 10 folds in different cell types studies with lowest activity in the range of 30nmol in HCT116 cells and highest activity in macrophages (~350 nmol).

In Vitro import of proteins into mitochondria

Mitochondria were isolated from control and ischemic hearts and used for *in vitro* import using a system described before (30–32). The import buffer contains 15 mM glutamate and 10 mM malate as energy source. The import assays were carried out in a 100-μl final volume and treated with 50 μg/ml trypsin for 15 min on ice, as described before (33). Trypsin-treated samples were mixed with a 10-fold excess of trypsin inhibitor, and mitochondria were recovered by sedimentation through 0.8M sucrose. Untreated mitochondrial suspension was diluted with mitochondrial isolation buffer and passed through 1 M sucrose. Resulting mitochondrial pellets were washed once with mitochondrial isolation buffer in the presence of 0.1 mM PMSF, and mitochondrial proteins were solubilized in 2× Laemmli buffer for 5 min at 95 °C and analyzed by SDS-PAGE. The gels were imaged through a STORM radiometric imager (Molecular Dynamics Inc).

Measuring PKA activity

Raw 264.7 mouse monocytes grown under hypoxia or normoxia were homogenized and mitochondria were isolated as described above and lysed in a detergent containing buffer provided in the kit. The lysate was clarified at 15,000 × g for 10 min and the supernatant was used for measuring the activity. Protein kinase A (PKA) activity was measured using the ELISA based PKA activity assay kit (Enzo Life Sciences, Villeubanne, France) according to manufacturer's instructions. Briefly, plates pre-coated with substrate were used. Each reaction contained 10μg of mitochondrial extract or cytosolic protein fractions and the reaction was initiated by adding ATP. The PKA activity was detected by using primary antibody and HRP-conjugated secondary antibody specific to the coated substrate. Active PKA provided in the kit was used as positive control and the assay buffer provided in the kit was used as negative control. Final reaction was measured by adding TMB substrate and the absorbance was measured at 450nm in a Chemeleon Multilabel detection platform. PKA inhibitor H89 (5μM) was added at the start of the reaction.

Chase of *in vitro* imported proteins

After the *in vitro* import of ^{35}S labeled WT and mutant CcO subunits, mitochondria were treated with 50 μg/ml of trypsin on ice for 15 minutes. Trypsin was inhibited by 10-fold excess of soybean trypsin inhibitor and mitochondria were passed through 0.8M sucrose cushion. Mitochondrial pellet was washed, and resuspended in 20mM HEPES-KOH pH 7.2/250 mM sucrose, 10 mM $MgCl_2$ and 2 mM ATP and incubated at 37oC for 60–90 min for chasing the ^{35}S label on CcO subunits. After the chase, samples were washed with the

incubation buffer, repelleted and proteins were resolved by SDS-PAGE. The ^{35}S labeled proteins were analyzed by imaging through the STORM (Molecular dynamics).

Transient transfection of Lon and flag-tagged CcO subunits in RAW 264.7 macrophages

Lon cDNA was cloned in mammalian expression vector PCDNA 3.1 and C-terminally FLAG tagged with intact targeting signals of CcO IVi1 and Vb cDNAs were cloned in pBABe Retroviral vector. Macrophages grown in Dulbecco's modified Eagle's medium to 40% confluence were co-transfected with Lon and flag-tagged CcO subunit cDNAs cloned in pCDNA vector (5μg DNA each/60mm plates) using Targefect, a non-liposomal transfection reagent (Targeting systems, CA) as per manufacturer's protocol. After 48h of transfection, cells were subjected to hypoxia as described above. Total cell proteins were extracted in 10 mM Tris-HCl pH 7.5 containing 1 mM EDTA and 1% SDS buffer and analyzed by SDS-PAGE and western blot.

Purification of recombinant proteins from *E. coli* cells

The human mitochondrial Lon was expressed and purified from the Rosetta *E. coli* strain (Novagen) as described previously (34). Briefly, bacteria producing an amino-terminal hexa histidine-tagged to Lon lacking its predicted mitochondrial targeting sequence were resuspended in Solution A (0.2 M NaCl, 25 mM Tris buffer, pH 7.5, 20% glycerol, 2 mM β-mercaptoethanol) and sonicated on ice. The lysate was centrifuged, and the supernatant was loaded onto a Ni^{2+}-NTA column (Qiagen). The column was washed three times with NTA buffer (0.5 M NaCl, 25 mM Tris, pH 7.5, 20% glycerol) and five times with Solution A containing 40 μM Imidazole. Lon bound to the column was eluted in three steps with Solution A containing 0.1, 0.2, or 0.3 M imidazole. Su9-DHFR DNA cloned in pET 28(a+) vector (Invitrogen), was expressed in *E. coli* BL21 (DE3) Codon Plus (RIL) cells and purified to 95% homogeneity as described above. The cDNAs encoding the mature mouse CcO subunits IVi1 and Vb (both WT, T40A Vb and T52A Vi1 mutants) were cloned into *E. coli* expression vector pET28(a+) to generate a hexa histidine tag at the N-termini. The cDNA constructs were expressed in *E. coli* BL21 (DE3) Codon Plus (RIL) (Stratagene) at 37°C for 3 hours in the presence of 1 mM isopropyl-1-thio-β-D-galactopyranoside. Wild type and mutant subunits were sequestered in insoluble inclusion bodies. The inclusion bodies were solubilized in 50 mM Tris-HCl, pH 8.0 containing 8 M urea and purified using a Ni^{2+}-NTA column (QIAGEN Inc.). The urea solubilized protein was refolded by dialyzing the protein for 3 hours in each of 50mM Tris-HCl pH8.0, 1 mM β-mercaptoethanol buffer containing 6M, 4M, 2M, 1M, 100 mM urea respectively with 0.075% deoxycholate. Finally, the storage buffer (20mM Tris-HCl, pH 8.0, 1 mM β-mercaptoethanol, 10% glycerol, 0.075% deoxycholate) without urea was used for dialysis.

Generation of stable CcO subunit IVi1 knockdown cell lines

shRNA targeted to CcO subunit IVi1 was cloned into pSilencer 2.0 vector. A sequence with no significant homology to any mouse transcript was also cloned and used as a control. C2C12 mouse skeletal myoblasts were maintained in DMEM supplemented with 10% FBS and 0.1% PenStrep. Cells were grown to 60% confluence in 6-well plates, and 2 μg of pSilencer plasmid per well (containing either CcO IVi1 or control shRNA) was transfected using Fugene HD according to the manufacturer's instructions. About 48h post transfection,

the cells were switched to selection media containing 250 μg/ml G418, 48 h after transfection and maintained for two weeks with media changes every three to four days. Selected colonies were analyzed for CcO IVi1 levels by both real-time PCR and Western blotting. For ectopic expression of CcO IVi1 subunit in the knockdown cells, wild type or S52A mutant cDNA was cloned in PMXS lentiviral vector. Every third base in the codons within the shRNA target sequence was replaced with degenerate bases by site directed mutagenesis (Quikchange site directed mutagenesis, Agilent), which rendered the mRNA resistant to stably expressing shRNA in these cells.

Three dimensional modeling of CcO complex and Lon complex

Three dimensional structural models of CcO showing spatial organization of phosphorylation sites of CcO IVi1 and Vb and models of Lon interaction with CcO subunits were developed based on the X-ray crystal structures of bovine CcO complex (35) and E. coli Lon protein (36;37) which shows close homology to mitochondrial Lon (38) using Swiss-Model software (39). The accuracy of the model was verified by energy calculations using Gramos 96 software. 3D visualization and rendering s were achieved by the Swiss PDB viewer.

Statistical Analysis

Data from cultured cells and isolated hearts are presented as means ± S. D. Differences between paired variables were determined using two-tailed Student's t tests for paired data. P values <0.05 were conside4red statistically significant.

Results

Increased expression of Lon during hypoxia and myocardial ischemia

In all the experiments reported here, hypoxia (0.2% O_2) for 6–12h and myocardial ischemia for 20 min (2.5ml/min flow rate) following a normal perfusion (37–42ml/min) for 1–2h were used. The immunoblot in Fig. 1A shows that the level of mitochondrial Lon was markedly increased in ischemic heart, while the level of mitochondrial TOM20, used as a loading control remained unaffected. The mitochondrial Lon level was also induced in RAW 264.7 macrophages grown under hypoxic condition for 12h (Fig 1B) compared to cells grown under normoxic condition. The level of CcO Vb and IVi1 were also reduced markedly when cells were exposed to hypoxia, which was partly reversed by exposure to normoxic condition (Fig. 1B). Figures 1C–E indicate the quantitation of band intensities for Lon, CcOIVi1 and Vb proteins under normoxia and hypoxia. Consistent with increased Lon protease level, the Lon mRNA was induced 2–3 fold in cells exposed 6h and 12h hypoxia. These results provide evidence for the induction of Lon protease in myocardial ischemia and cells grown under hypoxia. Results are also in line with a previous study showing increased expression of mitochondrial Lon under hypoxic conditions (40).

With a view to further evaluate the role of Lon in the degradation of CcO subunits during hypoxic stress, mitochondrial isolates from macrophages grown under different conditions described in Fig. 1B were assayed for CcO activity. The CcO activity (Fig. 2A) essentially reflected the subunit levels under hypoxic growth. It is seen that the CcO activity was

progressively reduced by hypoxia for 6h and 12h. The activity in cells co-transfected with Lon cDNA was markedly reduced in a time-dependent manner. The results suggest that Lon has a direct role in reduced CcO activity under hypoxia. Fig. 2B shows the mitochondrial and cytosolic PKA levels in macrophages grown under varying periods (zero to 12h) of hypoxia. It is seen that mitochondrial PKA increased nearly two fold by hypoxia for 6–12h while the cytosolic PKA levels reduced markedly in 3 to 9hr of hypoxia and returned to near control levels by 12h of hypoxia. Thus, the PKA levels in the two cellular compartments responded differently to hypoxic growth. Results (Fig. 2C) show that that the hypoxia induced mitochondrial PKA activity was effectively inhibited by 5μM H89, confirming the nature of PKA induced. Previous studies from our laboratory (4;26;27) showed increased PKAα catalytic subunit in mitochondria from ischemic rabbit and mouse hearts. Consistent with these results, Fig. 2D and E show that mitochondrial PKAα level is increased in RAW 264.7 macrophages grown under hypoxia for 6h and 12h. Notably, significant part of mitochondrial protein was resistant to limited protease digestion under conditions when cytosol exposed outermembrane protein TOM20 was degraded. These results confirm the activation of mitochondrial PKA under hypoxic growth condition.

Altered turnover of *in vitro* imported CcO subunits by ischemic heart mitochondria

Both CcO IVi1 and Vb are nuclear encoded subunits imported into mitochondria post-translationally from the cytoplasm. To determine whether the decreased subunit content during ischemia is due to reduced import efficiency of pre-proteins, mitochondria from control hearts and those subjected to ischemia were tested for protein import efficiency. We used SU9-DHFR, a protein, N-terminally fused to Neurospora crassa ATPase 9 signal, which is often used as a control for mitochondrial import of canonical signal containing proteins (41). Fig. 3A shows the import of WT and phosphorylation site mutant (S40A) subunit Vb. Following sedimentation through sucrose, mitochondria were digested with trypsin with or without added triton X-100. As seen, both WT and S40A mutant Vb were imported efficiently into isolated heart mitochondria. As expected, trypsin treatment following Triton X-100 treatment completely degraded the subunits confirming that the trypsin resistant protein is localized inside the mitochondrial inner membrane compartment. Fig. 3B shows the relative levels of protein import into mitochondria from control heart and hearts subjected to global ischemia for 20 min followed by 1h of reperfusion.

Results show that SU9-DHFR, and also both WT and S40A Vb subunits were imported at about similar levels. Further, the level of import of all three proteins was only marginally lower (~15%) in mitochondria from ischemic heart. These results suggest that impaired mitochondrial import may not be the only reason for the reported low subunit levels in ischemic mitochondria (4). Although not shown, WT and T52A CcO IVi1 subunits were imported at the same levels.

Since there was only a minor difference in the level of import of CcO subunits between the control and ischemic mitochondria, we determined the rates of turnover of imported WT Vb and mutant Vb proteins. Following import, mitochondria were washed, suspended in import buffer containing excess unlabeled Met, 15 mM each of malate and glutamate, and 50 mM ATP for different time periods. The residual ^{35}S labeled protein was quantified by SDS gel

electrophoresis of protein followed by radiographic imaging using the STORM system. Fluorograms in Fig. 4A show the levels of ^{35}S labeled WT and S40A mutant Vb proteins at various time periods of chase and the relative band intensities were plotted in Fig. 4B. It is seen that both the WT and mutant proteins are turned over at about the same rate in control mitochondria. However, in mitochondria from ischemic hearts, the WT protein was turned over at a much faster rate while the S40A mutant protein turned over at a rate comparable to the rates in control mitochondria. In Fig 4C, WT and T52A mutant CcO IVi1 proteins were imported into control heart and ischemic heart mitochondria similar to that in Fig. 4A and incubated up to 90 min to evaluate the turnover rates. The immunoblots show that the intensity of the WT IVi1 band remained nearly constant in control heart mitochondria, but steadily decreased in ischemic heart mitochondria. The intensity of T52A protein, on the other hand remained nearly constant in both control and ischemic mitochondria. These results suggest that phosphorylation of Vb at S40 and IVi1 at T52 positions render them more vulnerable for degradation.

Phosphorylation site mutant Vb and IVi1 subunits show resistance to Lon mediated degradation *in vivo*

The possible enhanced degradation of CcO subunits under hypoxia was further tested by ectopic expression of C-terminal Flag-tagged WT or phosphorylation site mutated CcO subunits Vb (S40A) and IVi1 (T52A) cDNA's in RAW 264.7 macrophage cells. Following transfection, cells were grown under normoxia or hypoxia for 12h and the protein levels were measured by immunoblot analysis. As seen from Fig 4D, Flag-tagged WT Vb subunit was degraded nearly completely (~80%) under hypoxia while a large part of the S40A mutant protein resisted degradation. Similarly, the Flag tagged WT subunit IVi1 was degraded markedly while the T52A mutant subunit IVi1 was not degraded under hypoxic condition (Fig. 4E). These results show that mutations at the putative PKA target sites markedly reduce hypoxia-induced degradation of both subunits IVi1 and Vb.

Degradation of CcO subunits by purified Lon *in vitro*

We next investigated if purified recombinant WT and S40A mutant CcO Vb proteins were degraded by purified Lon and also if PKA mediated phosphorylation of WT and mutant proteins affected their sensitivity to Lon mediated degradation under *in vitro* conditions. Both CcOIVi1 and CcOVb WT subunits are efficiently phosphorylated by PKA, though the level of phosphorylation was significantly lower in mutant subunits (Fig. S1). We carried out phosphorylation of Vb subunit formed in a wheat germ transcription-linked translation system, which is known to lack PKA activity. Fig. S1A shows that His-tagged WT and S4A subunits of Vb are translated at a comparable level in the wheat germ system. Fig. S1B and C show that S40A subunit is phosphorylated at about 20–25% reduced level than the WT. Both the WT and mutant proteins were phosphorylated even in the absence of added PKA suggesting that the protein is phosphorylated by other wheat germ resident kinases.

To determine whether the phosphorylated subunits are substrates for Lon mediated proteolysis, purified CcO subunits were incubated *in vitro* with purified recombinant Lon protein with or without added PKA and ATP. Figure 5A shows the gel profile of typical *in vitro* reaction mixtures with WT or S40A mutant Vb and recombinant Lon. The gel pattern

at the top (Fig. 5A) represents the stained gel showing the relative purity of CcO Vb subunits and the Lon protein used in the assay. The lower two profiles are immunoblots developed with antibodies to Lon and CcOVb proteins, respectively. Results show no major contaminating proteins in the proteins used for reconstitution. Figure 5B shows the time course of degradation of WT and S40A proteins with or without phosphorylation. Reactions were run as in Figure 5A for zero to 90 min and the relative band intensities of Vb protein were determined by image analysis. It is seen that unphosphorylated WT as well as Mut Vb proteins were not degraded to any significant level up to 90 min. The phosphorylated Vb protein, on the other hand, was degraded in a time dependent manner. Interestingly, the phosphorylation resistant S40A mutant protein was not degraded even after preincubation with added PKA. These results provide direct evidence that phosphorylated Vb protein serves as a more efficient substrate for Lon mediated degradation. As shown in Fig. 5C, WT subunit IVi1 was degraded when PKA, ATP and purified Lon were added to the reaction mixture, while the T52A mutant protein was resistant to degradation under these conditions. Our results therefore confirm the role of Lon in the degradation of phosphorylated subunits of CcO complex and also reveal the importance of phosphorylation sites S40 of Vb and T52 of IVi1 subunits in Lon mediated degradation.

CcO subunits are resistant to hypoxia in Lon depleted cells

To directly implicate the function of Lon in the turnover of CcO subunits, Lon mRNA was silenced in human colon carcinoma cells by stable expression of antisense or non-specific oligonucleotides (42). To ensure the generality of Lon mediated degradation of CcO subunits, we used human colon carcinoma cell line LS174T in this experiment. Control and Lon depleted cells were grown under normoxia or hypoxia for 12h and the levels of CcO subunits were analyzed. It is seen from Fig 6A that there was no detectable Lon in siRNA expressing cells; however, the immunoblot shows detectable Lon in non-specific siRNA expressing cells (control cells). Results also show no detectable reduction in subunit IVi1 following hypoxia in Lon depleted cells. However, the level of IVi1 protein was markedly reduced in control cells following hypoxia. These results suggest that Lon has an essential function in the degradation of CcOVb and CcOIVi1 subunits during hypoxia and probably plays a role in the turnover of these subunits during normoxic conditions as well.

Consistent with the decline in subunit levels, there was a significant reduction in the CcO activity in hypoxic cells (Fig 6B). In contrast, there is heightened CcO activity in Lon depleted control cells, both under normoxic and hypoxic growth conditions. Notably, hypoxia mediated decline in CcO activity was not observed in Lon depleted cells. These results provide direct evidence for the role of Lon protease in the modulation of CcO subunits as well as activity under hypoxia.

Stable expression of S52A mutant subunit of CcO IVi1 restores the CcO activity under hypoxic condition

To further assess the role of phosphorylation in the selective degradation of subunit IVi1, we first generated a cell line stably expressing shRNA against CcO subunit IVi1 (C4KD). We then ectopically expressed either wild type (WT-shR) or phosphorylation resistant subunit IVi1 cDNA (S52A-shR) IV in the C4KD cells. To prevent shRNA mediated degradation in

C4KD cells, the shRNA target region of subunit IVi1 cDNA was modified with degenerate codons. The effect of hypoxia on subunit IVi1 levels in these cells is shown in Fig 7A. Hypoxia induced a reduction in subunit IVi1 level by more than 50% in control cells and nearly 75% in C4KD cells reconstituted with wild type subunit IVi1. SDHA, which is unaffected by hypoxia was used as a loading control. Notably, in cells reconstituted with S52A-mutant, hypoxia caused only about 25% loss of subunit IVi1. We next tested the effects of hypoxia on CcO activities in these cells. As shown in Fig. 7B, CcO activity was reduced by about 50–60% in both control and WT-shR cells exposed to hypoxia, whereas the activity was reduced by only about 20% in cells reconstituted phosphorylation resistant S52A mutant following hypoxia (Fig 7B). These results confirm that the S52A mutant subunit of IVi1 is relatively more resistant to hypoxia mediated damage and that phosphorylation of subunits is responsible for hypoxia mediated loss of CcO activity

Localization of phosphorylation sites of CcO IVi1 and Vb and their accessibility to soluble matrix proteins

We generated a 3D structural model based on the reported crystal structure of bovine CcO complex (35) as described before (10). The model in Fig. 8A shows that both the S52 of CcOIVi1 and S40 of Vb (shown with red arrows) are positioned in a loop out structure in the aqueous environment of the matrix. These sites should be easily accessible to mitochondrial PKA for phosphorylation. We also generated a 3D model of bacterial Lon to understand if the complex can accommodate CcOVb and IVi1 subunits under energy minimization conditions. The models in Fig. 8B and C show that both CcOVb and IVi1 structures can be docked on to Lon complex under energy minimization conditions. The lateral views show that both proteins protrude deep into Lon pocket gaining access to the Lon catalytic domain. These results further suggest feasibility of Lon mediated degradation of CcO subunits.

Discussion

Hypoxia and ischemia injury often result in oxidative stress and mitochondrial impairment (43). Impaired mitochondrial function and oxidative stress lead to brain dysfunction and neuronal death during neonatal and adult stages of the human life cycle (44). Several studies suggest that CcO, the terminal oxidase of the mitochondrial electron transfer chain is damaged during ischemia (2;8;12;17;45). Mechanisms of functional impairment of CcO under these pathophysiological conditions have been the subject of investigation by several groups. Studies by Lesnefsky's laboratory showed that altered cardiolipin composition of innermembrane plays critical role in altered CcO activity (45). Studies from our laboratory implicated PKA-mediated phosphorylation and degradation of subunits I, IVi1 and Vb during hypoxia and ischemia as possible cause of altered CcO structure and function (4). PKA inhibitors, H89 restored the activity as well as subunit content (4). In this report we further evaluated the mechanisms by which the phosphorylated subunits of CcO IVi1 and Vb are degraded. Our results show that Lon protease plays a major role in the turnover of CcO subunits during hypoxia and myocardial ischemia.

Lon belongs to AAA protease family and is highly conserved from bacteria to humans. It has been shown that mitochondrial matrix from liver and heart contains proteolytic activity

that degrades oxidized, dysfunctional, and misfolded proteins including mitochondrial aconitase (46). Later it was found that Lon mediates the degradation of oxidized and misfolded proteins. Pim1, the Lon homologue in yeast, is involved in the maintenance of mitochondrial DNA nucleoids and is required for intra-mitochondrial proteolysis (47;48). Lon deletion often results in loss of mitochondrial DNA in yeast (48). The Lon protease contains three domains: a) N-terminal domain that is required for interacting with protein substrates, b) AAA+ module that consists of ATP binding and ATP hydrolysis domain, and c) the C-terminal domain with serine and lysine active site residues that form the catalytic dyad which is essential for proteolytic activity (48;49).

Several recent studies have implicated mitochondrial ATP dependent proteases in pathological and stressful conditions. It has been shown that ATP dependent proteolytic activity increases in cardiac ischemia--reperfusion (50). The Lon activity is increased in rats bearing Zajdela hepatoma or in T3-treated hypothyroid animals (51). Some reports suggest increased Lon promoter activity as well as Lon antigen levels during hypoxia and ischemia/reperfusion stress (50;52). Our findings suggest that in both macrophages exposed to hypoxia and rabbit hearts subjected to ischemia, mitochondrial Lon protein as well as mRNA levels are increased in a time-dependent manner (Figure 1). Results also show forcible overexpression of Lon induces CcO subunit degradation similar to that observed in cells grown under hypoxia and hearts subjected to ischemia/reperfusion thus confirming the role of Lon in this process. It is known that mitochondrial calpain activated under pathological conditions is involved in protein degradation causing mitochondrial dysfunction (53). While several factors may be involved in eliciting hypoxia and ischemic damage, our results clearly show that increased mitochondrial PKA activity and increased Lon activity are critically important for CcO disruption observed in hypoxia and myocardial ischemia/reperfusion.

Studies suggest that Lon modulates the level of mitochondrial Tfam, which plays multiple roles in mitochondrial transcription, replication, mtDNA maintenance and organization of nucleoid structures (54–56). Initially Kaguni's laboratory (55) showed that Lon protease regulates mtDNA copy number possibly through degradation of Tfam (mitochondrial transcription factor A). More recently Lu et al. (44) showed phosphorylation of Tfam not only alters its DNA binding ability, but also its sensitivity to Lon mediated degradation (54) under conditions when catalytic subunit of PKAα was forcibly expressed in mitochondria. Studies from our laboratory showed that PKA mediated phosphorylation of CcO subunits I, IVi1 and Vb during pathophysiological conditions of hypoxia and myocardial ischemia/reperfusion renders them more sensitive to degradation (4, 8–10, 26–28). In support, Hori et al. showed that ER stress transmitted to mitochondria also induce degradation of CcO II, IVi1 and Vb in a Lon dependent manner (52). The present study extends these observations in multiple ways: First, we provide additional data on the increased mitochondrial PKAα and increased PKA activity in RAW macrophages grown under hypoxia. Currently the mechanism of import of PKA subunits into mitochondria or its activation/inactivation remains unclear. Additional studies will be required to understand the mechanism. Mitochondrial PKA activated under these pathophysiological conditions is not induced by mitochondrial adenyl cyclase-cAMP, but by mitochondrial redox state (27). Second, in continuation of previous studies, we now show that mitochondrial PKA induced under

hypoxia and myocardial ischemia induces the degradation of CcO subunits IVi1 and Vb resulting in marked inhibition of CcO activity. Third, similar to that of the role of phosphorylation in ubiquitin mediated protein degradation, our results for the first time suggest that phosphorylation CcO subunits may provide signal for their Lon mediated degradation.

Results presented in this paper support the view that Lon is involved in the turnover of CcO subunits: First, we observed a time dependent decrease in subunit content and CcO activity in cells with increased Lon protein during hypoxic stress (Figure 1). Second, *in vitro* mitochondrial import studies and transient expression of flag-tagged CcO subunits show that phosphorylated IVi1 and Vb proteins are preferentially degraded under hypoxia, and myocardial ischemia. Additionally use of in vitro imported proteins in isolated heart mitochondria show that WT subunits are more rapidly degraded in ischemic heart mitochondria than phosphorylation site mutant proteins.

Lon protease requires several other factors in addition to ATP. It has been shown that in E.coli, Lon protease activity and the substrate preference is modulated by inorganic polyphosphate for the degradation of ribosomal S2 protein (57). Also, Lon has been shown to degrade folded or misfolded proteins non-processively (50;58). Some reports suggest that Lon selectively degrades the oxidized form of aconitase in human Wi-38 lung fibroblasts (59). Nevertheless, ours is probably the first study showing that phosphorylated subunits of CcO are the substrates for Lon protease during hypoxia and myocardial ischemia injury. To further support our hypothesis, the murine S40A mutant of CcOVb and S52A mutant of CcO IVi1 imported into ischemic mitochondria *in vitro* are more stable compared to wild type protein in a chase reaction (Fig. 4A and B). Our results further demonstrate that these phosphorylation site mutants are protected from hypoxic stress even in the presence of over-expressed Lon (Fig. 5). Our results in conjunction with the results of Lu et al. (44) confirm that in addition to oxidized and unfolded proteins, several phosphorylated proteins also serve as specific substrates of Lon. Consistent with this, our 3D model (Figure 8A) indeed shows that the phosphorylation sites of both Vb and IVi1 are exposed to the matrix amenable for interaction with soluble PKA enzyme.

Lon depletion in iWI-38 VA-13 human lung fibroblasts results in caspase 3 activation and apoptotic death. The surviving cells show abnormal mitochondrial function and morphology and eventually are lost by necrosis (60). Lon is known to have several mitochondrial targets; it is likely that long term depletion of Lon results in altered function/activity of many proteins and possible loss of mtDNA. We find that Lon depleted human HS 189 cells show abnormal morphology and slow growth but not apoptotic death (unpublished results). Our results may lead to the design of more selective inhibitors of mitochondrial Lon protease and or PKA enzyme to minimize the hypoxic and ischemic damage to cells. It is not clear if phosphorylated subunits are released from the complex before degradation or if Lon protease is recruited by phosphorylated subunits while associated with the complex. Currently experiments are underway to address these questions. It is nevertheless apparent from 3D modeling (Figure 8C and D) that both of the CcO subunits under study can associate with the Lon protease under energy minimization conditions. We therefore describe a novel role of Lon protease in modulating mitochondrial function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Reference List

1. Lee I, Bender E, Kadenbach B. Control of mitochondrial membrane potential and ROS formation by reversible phosphorylation of cytochrome c oxidase. Mol Cell Biochem. 2002 May; 234–235(1–2): 63–70.
2. Lesnefsky EJ, Gudz TI, Migita CT, Ikeda-Saito M, Hassan MO, Turkaly PJ, et al. Ischemic Injury to Mitochondrial Electron Transport in the Aging Heart: Damage to the Iron-Sulfur Protein Subunit of Electron Transport Complex III. Archives of Biochemistry and Biophysics. 2001 Jan 1; 385(1):117–28. [PubMed: 11361007]
3. Ludwig B, Bender E, Arnold S, Huttemann M, Lee I, Kadenbach B. Cytochrome C oxidase and the regulation of oxidative phosphorylation. Chembiochem. 2001 Jun 1; 2(6):392–403. [PubMed: 11828469]
4. Prabu SK, Anandatheerthavarada HK, Raza H, Srinivasan S, Spear JF, Avadhani NG. Protein kinase A-mediated phosphorylation modulates cytochrome c oxidase function and augments hypoxia and myocardial ischemia-related injury. J Biol Chem. 2006 Jan 27; 281(4):2061–70. [PubMed: 16303765]
5. Traaseth N, Elfering S, Solien J, Haynes V, Giulivi C. Role of calcium signaling in the activation of mitochondrial nitric oxide synthase and citric acid cycle. Biochim Biophys Acta. 2004 Jul 23; 1658(1–2):64–71. [PubMed: 15282176]
6. Helling S, Huttemann M, Ramzan R, Kim SH, Lee I, Muller T, et al. Multiple phosphorylations of cytochrome c oxidase and their functions. Proteomics. 2012 Apr; 12(7):950–9. [PubMed: 22522801]
7. Lee I, Salomon AR, Ficarro S, Mathes I, Lottspeich F, Grossman LI, et al. cAMP-dependent Tyrosine Phosphorylation of Subunit I Inhibits Cytochrome c Oxidase Activity. J Biol Chem. 2005 Feb 18; 280(7):6094–100. [PubMed: 15557277]
8. Vijayasarathy C, Damle S, Lenka N, Avadhani NG. Tissue variant effects of heme inhibitors on the mouse cytochrome c oxidase gene expression and catalytic activity of the enzyme complex. Eur J Biochem. 1999 Nov; 266(1):191–200. [PubMed: 10542064]
9. Vijayasarathy C, Damle S, Prabu SK, Otto CM, Avadhani NG. Adaptive changes in the expression of nuclear and mitochondrial encoded subunits of cytochrome c oxidase and the catalytic activity during hypoxia 1. Eur J Biochem. 2003 Mar; 270(5):871–9. [PubMed: 12603320]
10. Fang JK, Prabu SK, Sepuri NB, Raza H, Anandatheerthavarada HK, Galati D, et al. Site specific phosphorylation of cytochrome c oxidase subunits I, IVi1 and Vb in rabbit hearts subjected to ischemia/reperfusion. FEBS Letters. 2007 Apr 3; 581(7):1302–10. [PubMed: 17349628]
11. Acin-Perez R, Gatti DL, Bai Y, Manfredi G. Protein phosphorylation and prevention of cytochrome oxidase inhibition by ATP: coupled mechanisms of energy metabolism regulation. Cell Metab. 2011 Jun 8; 13(6):712–9. [PubMed: 21641552]
12. Srinivasan S, Avadhani NG. Cytochrome c oxidase dysfunction in oxidative stress. Free Radic Biol Med. 2012 Sep 15; 53(6):1252–63. [PubMed: 22841758]
13. Huttemann M, Helling S, Sanderson TH, Sinkler C, Samavati L, Mahapatra G, et al. Regulation of mitochondrial respiration and apoptosis through cell signaling: cytochrome c oxidase and cytochrome c in ischemia/reperfusion injury and inflammation. Biochim Biophys Acta. 2012 Apr; 1817(4):598–609. [PubMed: 21771582]

14. Paradies G, Ruggiero FM, Petrosillo G, Quagliariello E. Age-dependent decrease in the cytochrome c oxidase activity and changes in phospholipids in rat-heart mitochondria. Arch Gerontol Geriatr. 1993 May; 16(3):263–72. [PubMed: 15374339]
15. Paradies G, Petrosillo G, Pistolese M, Ruggiero FM. The effect of reactive oxygen species generated from the mitochondrial electron transport chain on the cytochrome c oxidase activity and on the cardiolipin content in bovine heart submitochondrial particles. FEBS Letters. 2000 Jan 28; 466(2–3):323–6. [PubMed: 10682852]
16. Sohal RS. Aging, cytochrome oxidase activity, and hydrogen peroxide release by mitochondria. Free Radic Biol Med. 1993 Jun; 14(6):583–8. [PubMed: 8392019]
17. Lesnefsky EJ, Tandler B, Ye J, Slabe TJ, Turkaly J, Hoppel CL. Myocardial ischemia decreases oxidative phosphorylation through cytochrome oxidase in subsarcolemmal mitochondria. Am J Physiol. 1997 Sep; 273(3 Pt 2):H1544–H1554. [PubMed: 9321848]
18. Piper HM, Sezer O, Schleyer M, Schwartz P, Hutter JF, Spieckermann PG. Development of ischemia-induced damage in defined mitochondrial subpopulations. J Mol Cell Cardiol. 1985 Sep; 17(9):885–96. [PubMed: 4046049]
19. Ueta H, Ogura R, Sugiyama M, Kagiyama A, Shin GO2- spin trapping on cardiac submitochondrial particles isolated from ischemic and non-ischemic myocardium. J Mol Cell Cardiol. 1990 Aug; 22(8):893–9. [PubMed: 2172558]
20. Srinivasan S, Guha M, Dong DW, Whelan KA, Ruthel G, Uchikado Y, et al. Disruption of cytochrome c oxidase function induces the Warburg effect and metabolic reprogramming. Oncogene. 2015 Jul 6.
21. Oliva CR, Markert T, Gillespie GY, Griguer CE. Nuclear-encoded cytochrome c oxidase subunit 4 regulates BMI1 expression and determines proliferative capacity of high-grade gliomas. Oncotarget. 2015 Feb 28; 6(6):4330–44. [PubMed: 25726526]
22. Itoh K, Weis S, Mehraein P, Muller-Hocker J. Defects of cytochrome c oxidase in the substantia nigra of Parkinson's disease: and immunohistochemical and morphometric study. Mov Disord. 1997 Jan; 12(1):9–16. [PubMed: 8990048]
23. Arnold S. Cytochrome c oxidase and its role in neurodegeneration and neuroprotection. Adv Exp Med Biol. 2012; 748:305–39. [PubMed: 22729864]
24. Boveris A, Cadenas E. Mitochondrial production of hydrogen peroxide regulation by nitric oxide and the role of ubisemiquinone. IUBMB Life. 2000 Oct; 50(4–5):245–50. [PubMed: 11327317]
25. Chen Q, Lesnefsky EJ. Depletion of cardiolipin and cytochrome c during ischemia increases hydrogen peroxide production from the electron transport chain. Free Radical Biology and Medicine. 2006 Mar 15; 40(6):976–82. [PubMed: 16540393]
26. Galati D, Srinivasan S, Raza H, Prabu SK, Hardy M, Chandran K, et al. Role of nuclear-encoded subunit Vb in the assembly and stability of cytochrome c oxidase complex: implications in mitochondrial dysfunction and ROS production. Biochem J. 2009 Jun 15; 420(3):439–49. [PubMed: 19338496]
27. Srinivasan S, Spear J, Chandran K, Joseph J, Kalyanaraman B, Avadhani NG. Oxidative stress induced mitochondrial protein kinase A mediates cytochrome c oxidase dysfunction. PLoS One. 2013; 8(10):e77129. [PubMed: 24130844]
28. Spear JF, Moore EN. Preconditioning attenuates the shortening of recovery during coronary occlusion in isolated rabbit hearts with D-sotalol-induced long QT intervals. J Cardiovasc Pharmacol. 2002 May; 39(5):761–76. [PubMed: 11973421]
29. Bhat NK, Niranjan BG, Avadhani NG. Qualitative and comparative nature of mitochondrial translation products in mammalian cells. Biochemistry. 1982 May 11; 21(10):2452–60. [PubMed: 7093198]
30. Sepuri NB, Schulke N, Pain D. GTP hydrolysis is essential for protein import into the mitochondrial matrix. J Biol Chem. 1998 Jan 16; 273(3):1420–4. [PubMed: 9430677]
31. Sepuri NB, Gordon DM, Pain D. A GTP-dependent "push" is generally required for efficient protein translocation across the mitochondrial inner membrane into the matrix. J Biol Chem. 1998 Aug 14; 273(33):20941–50. [PubMed: 9694843]

32. Tammineni P, Anugula C, Mohammed F, Anjaneyulu M, Larner AC, Sepuri NB. The import of the transcription factor STAT3 into mitochondria depends on GRIM-19, a component of the electron transport chain. J Biol Chem. 2013 Feb 15; 288(7):4723–32. [PubMed: 23271731]
33. Addya S, Anandatheerthavarada HK, Biswas G, Bhagwat SV, Mullick J, Avadhani NG. Targeting of NH2-terminal-processed microsomal protein to mitochondria: a novel pathway for the biogenesis of hepatic mitochondrial P450MT2. J Cell Biol. 1997 Nov 3; 139(3):589–99. [PubMed: 9348277]
34. Lu B, Liu T, Crosby JA, Thomas-Wohlever J, Lee I, Suzuki CK. The ATP-dependent Lon protease of Mus musculus is a DNA-binding protein that is functionally conserved between yeast and mammals. Gene. 2003 Mar 13.306:45–55. [PubMed: 12657466]
35. Tsukihara T, Aoyama H, Yamashita E, Tomizaki T, Yamaguchi H, Shinzawa-Itoh K, et al. The whole structure of the 13-subunit oxidized cytochrome c oxidase at 2.8 A. Science. 1996 May 24; 272(5265):1136–44. [PubMed: 8638158]
36. Cha SS, An YJ, Lee CR, Lee HS, Kim YG, Kim SJ, et al. Crystal structure of Lon protease: molecular architecture of gated entry to a sequestered degradation chamber. EMBO J. 2010 Oct 20; 29(20):3520–30. [PubMed: 20834233]
37. Botos I, Melnikov EE, Cherry S, Khalatova AG, Rasulova FS, Tropea JE, et al. Crystal structure of the AAA+ alpha domain of E. coli Lon protease at 1.9A resolution. J Struct Biol. 2004 Apr; 146(1–2):113–22. [PubMed: 15037242]
38. Wang N, Gottesman S, Willingham MC, Gottesman MM, Maurizi MR. A human mitochondrial ATP-dependent protease that is highly homologous to bacterial Lon protease. Proc Natl Acad Sci U S A. 1993 Dec 1; 90(23):11247–51. [PubMed: 8248235]
39. Guex N, Peitsch MC. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis. 1997 Dec; 18(15):2714–23. [PubMed: 9504803]
40. Fukuda R, Zhang H, Kim Jw, Shimoda L, Dang CV, Semenza GL. HIF-1 Regulates Cytochrome Oxidase Subunits to Optimize Efficiency of Respiration in Hypoxic Cells. Cell. 2007 Apr 6; 129(1):111–22. [PubMed: 17418790]
41. Geissler A, Krimmer T, Bomer U, Guiard B, Rassow J, Pfanner N. Membrane potential-driven protein import into mitochondria. The sorting sequence of cytochrome b(2) modulates the deltapsi-dependence of translocation of the matrix-targeting sequence. Mol Biol Cell. 2000 Nov; 11(11): 3977–91. [PubMed: 11071921]
42. Lu B, Yadav S, Shah PG, Liu T, Tian B, Pukszta S, et al. Roles for the human ATP-dependent Lon protease in mitochondrial DNA maintenance. J Biol Chem. 2007 Jun 15; 282(24):17363–74. [PubMed: 17420247]
43. Blomgren K, Hagberg H. Free radicals, mitochondria, and hypoxia-ischemia in the developing brain. Free Radic Biol Med. 2006 Feb 1; 40(3):388–97. [PubMed: 16443153]
44. Koh HS, Chang CY, Jeon SB, Yoon HJ, Ahn YH, Kim HS, et al. The HIF-1/glial TIM-3 axis controls inflammation-associated brain damage under hypoxia. Nat Commun. 2015; 6:6340. [PubMed: 25790768]
45. Lesnefsky EJ, Chen Q, Slabe TJ, Stoll MS, Minkler PE, Hassan MO, et al. Ischemia, rather than reperfusion, inhibits respiration through cytochrome oxidase in the isolated, perfused rabbit heart: role of cardiolipin. Am J Physiol Heart Circ Physiol. 2004 Jul; 287(1):H258–H267. [PubMed: 14988071]
46. Marcillat O, Zhang Y, Lin SW, Davies KJ. Mitochondria contain a proteolytic system which can recognize and degrade oxidatively-denatured proteins. Biochem J. 1988 Sep 15; 254(3):677–83. [PubMed: 3196285]
47. van DL, Pearce DA, Sherman F. PIM1 encodes a mitochondrial ATP-dependent protease that is required for mitochondrial function in the yeast Saccharomyces cerevisiae. J Biol Chem. 1994 Jan 7; 269(1):238–42. [PubMed: 8276800]
48. Suzuki CK, Suda K, Wang N, Schatz G. Requirement for the yeast gene LON in intramitochondrial proteolysis and maintenance of respiration. Science. 1994 Apr 8; 264(5156):273–6. [PubMed: 8146662]

49. Amerik AI, Antonov VK, Ostroumova NI, Rotanova TV, Chistiakova LG. [Cloning, structure and expression of the full-size lon gene in Escherichia coli coding for ATP-dependent La-proteinase]. Bioorg Khim. 1990 Jul; 16(7):869–80. [PubMed: 2242054]
50. Bulteau AL, Lundberg KC, Ikeda-Saito M, Isaya G, Szweda LI. Reversible redox-dependent modulation of mitochondrial aconitase and proteolytic activity during in vivo cardiac ischemia/reperfusion. Proc Natl Acad Sci U S A. 2005 Apr 26; 102(17):5987–91. [PubMed: 15840721]
51. Luciakova K, Sokolikova B, Chloupkova M, Nelson BD. Enhanced mitochondrial biogenesis is associated with increased expression of the mitochondrial ATP-dependent Lon protease. FEBS Lett. 1999 Feb 12; 444(2–3):186–8. [PubMed: 10050756]
52. Hori O, Ichinoda F, Tamatani T, Yamaguchi A, Sato N, Ozawa K, et al. Transmission of cell stress from endoplasmic reticulum to mitochondria: enhanced expression of Lon protease. J Cell Biol. 2002 Jun 24; 157(7):1151–60. [PubMed: 12082077]
53. Thompson J, Hu Y, Lesnefsky EJ, Chen Q. Activation of mitochondrial calpain and increased cardiac injury: beyond AIF release. Am J Physiol Heart Circ Physiol. 2016 Feb 1; 310(3):H376–H384. [PubMed: 26637561]
54. Lu B, Lee J, Nie X, Li M, Morozov YI, Venkatesh S, et al. Phosphorylation of human TFAM in mitochondria impairs DNA binding and promotes degradation by the AAA+ Lon protease. Mol Cell. 2013 Jan 10; 49(1):121–32. [PubMed: 23201127]
55. Matsushima Y, Goto Y, Kaguni LS. Mitochondrial Lon protease regulates mitochondrial DNA copy number and transcription by selective degradation of mitochondrial transcription factor A (TFAM). Proc Natl Acad Sci U S A. 2010 Oct 26; 107(43):18410–5. [PubMed: 20930118]
56. Venkatesh S, Lee J, Singh K, Lee I, Suzuki CK. Multitasking in the mitochondrion by the ATP-dependent Lon protease. Biochim Biophys Acta. 2012 Jan; 1823(1):56–66. [PubMed: 22119779]
57. Nishii W, Suzuki T, Nakada M, Kim YT, Muramatsu T, Takahashi K. Cleavage mechanism of ATP-dependent Lon protease toward ribosomal S2 protein. FEBS Lett. 2005 Dec 19; 579(30):6846–50. [PubMed: 16337203]
58. Ondrovicova G, Liu T, Singh K, Tian B, Li H, Gakh O, et al. Cleavage site selection within a folded substrate by the ATP-dependent lon protease. J Biol Chem. 2005 Jul 1; 280(26):25103–10. [PubMed: 15870080]
59. Bota DA, Davies KJ. Lon protease preferentially degrades oxidized mitochondrial aconitase by an ATP-stimulated mechanism. Nat Cell Biol. 2002 Sep; 4(9):674–80. [PubMed: 12198491]
60. Bota DA, Ngo JK, Davies KJ. Downregulation of the human Lon protease impairs mitochondrial structure and function and causes cell death. Free Radic Biol Med. 2005 Mar 1; 38(5):665–77. [PubMed: 15683722]

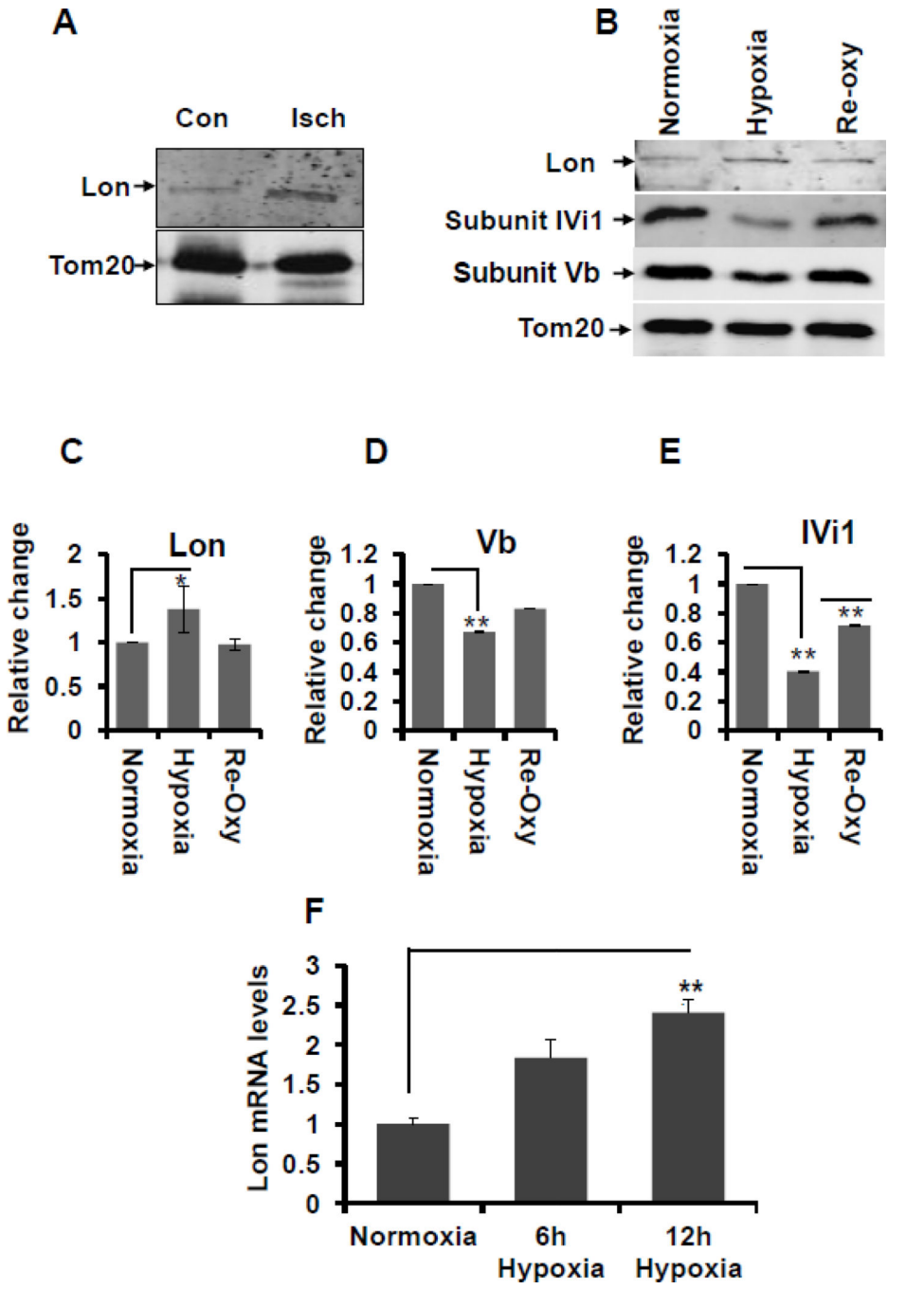

Figure 1.

Reciprocal levels of mitochondrial Lon and CcO subunits under hypoxia and myocardial ischemia: a) Mitochondrial proteins from control heart and rabbit hearts subjected to global ischemia for 20 min/reperfusion for 1h were analyzed by immunoblot analysis using antibody to Lon protein. Tom20 antibody was used to assess the loading levels. b) mitochondrial proteins from macrophages grown under normoxia (Nor), hypoxia (Hyp) for 12h (0.2% O_2) or reoxygenation for 6h following hypoxia for 12h were subjected to immunoblot analysis using antibodies to Lon, CcO Vb and IVi1 proteins. Tom 20 antibody

was used for assessing the protein loading level. 30μg protein was loaded in each well. Relative band intensities for Lon, Vb and IVi1 proteins are presented in C, D, and E, respectively. The values represent mean±SD (n=3). The band intensity of normoxia sample was considered as 1. Fig. F shows the relative levels of Lon mRNA in cells grown under normoxia for 12h and hypoxia for 6h and 12h as shown. The values represent Mean ± SD from 3 separate estimates.

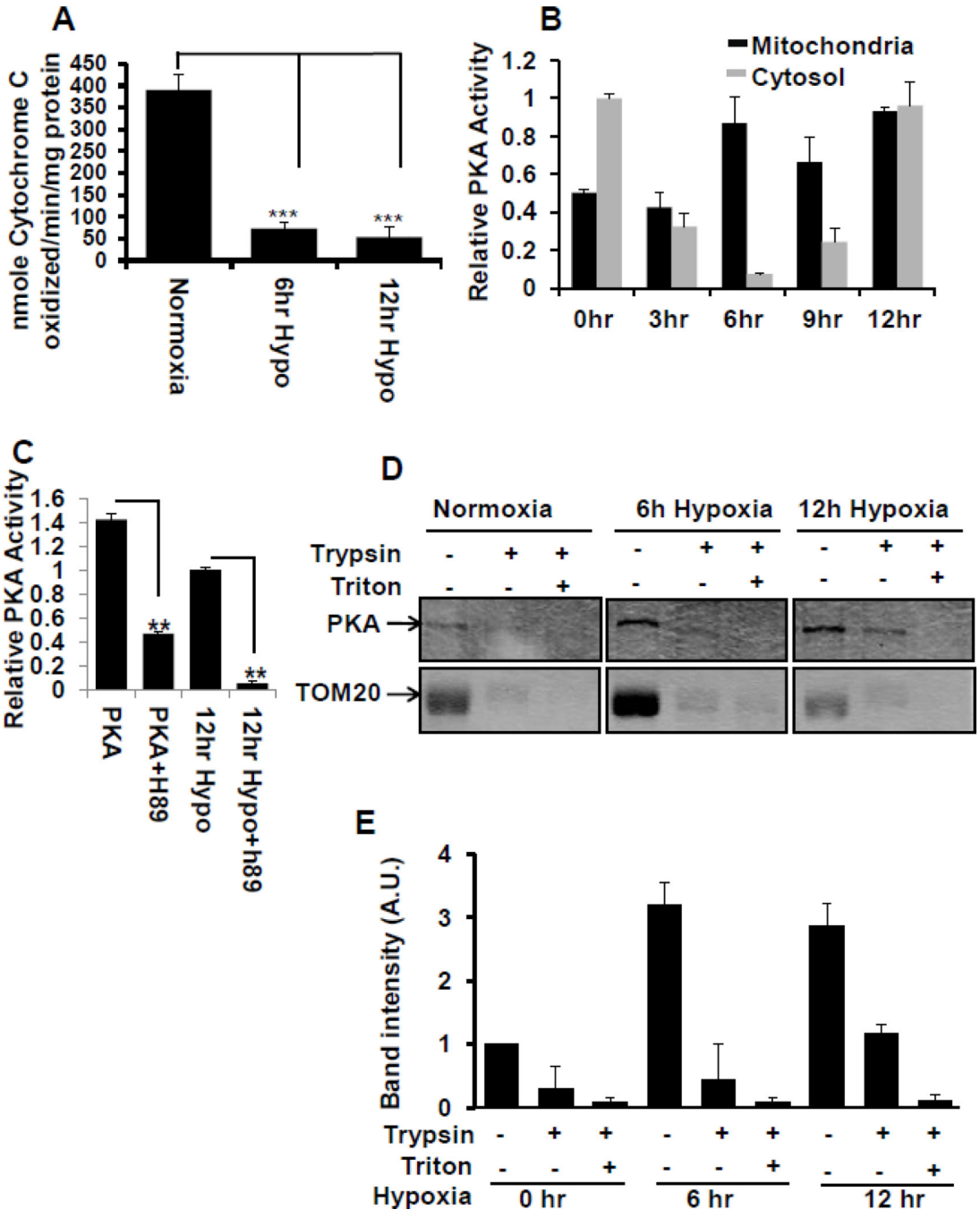

Figure 2.

Hypoxia mediated inhibition of CcO activity and induction of mitochondrial PKA in RAW264.7 cells: A) Mitochondrial isolates from cells grown under normoxia (Nor) and hypoxia for 6 or 12h were used for assaying CcO activity. B) PKA activity in the mitochondrial and cytosolic fractions from control cells or following hypoxia for 3, 6, 9 and 12h was measured with 10 μg protein as described in the Materials and Methods. Relative PKA activity was calculated by taking activity of 0h cytosolic fraction as 1. C) A control experiment showing that 12h hypoxia induced mitochondrial PKA is sensitive to 5μM H89

added at the start of the reaction. D and E) Mitochondria from cells grown under hypoxia for zero, 6h and 12h were treated with trypsin (7.5 μg/100 mg protein for 10 min on ice) or without trypsin as indicated in the Figure. In some cases, mitochondria were treated with 0.2% triton X-100 before treating with trypsin. Proteins were resolved on 12% SDS-PAGE and subjected to immunoblot analysis with antibody to PKAα as described in the Materials and Methods section. Antibody to TOM 20 was used as a control for outermembrane protein. The relative band intensities presented in D show a time dependent increase in trypsin resistant PKA protein. In all cases the results represent mean ± SD from 3–4 separate experiments.

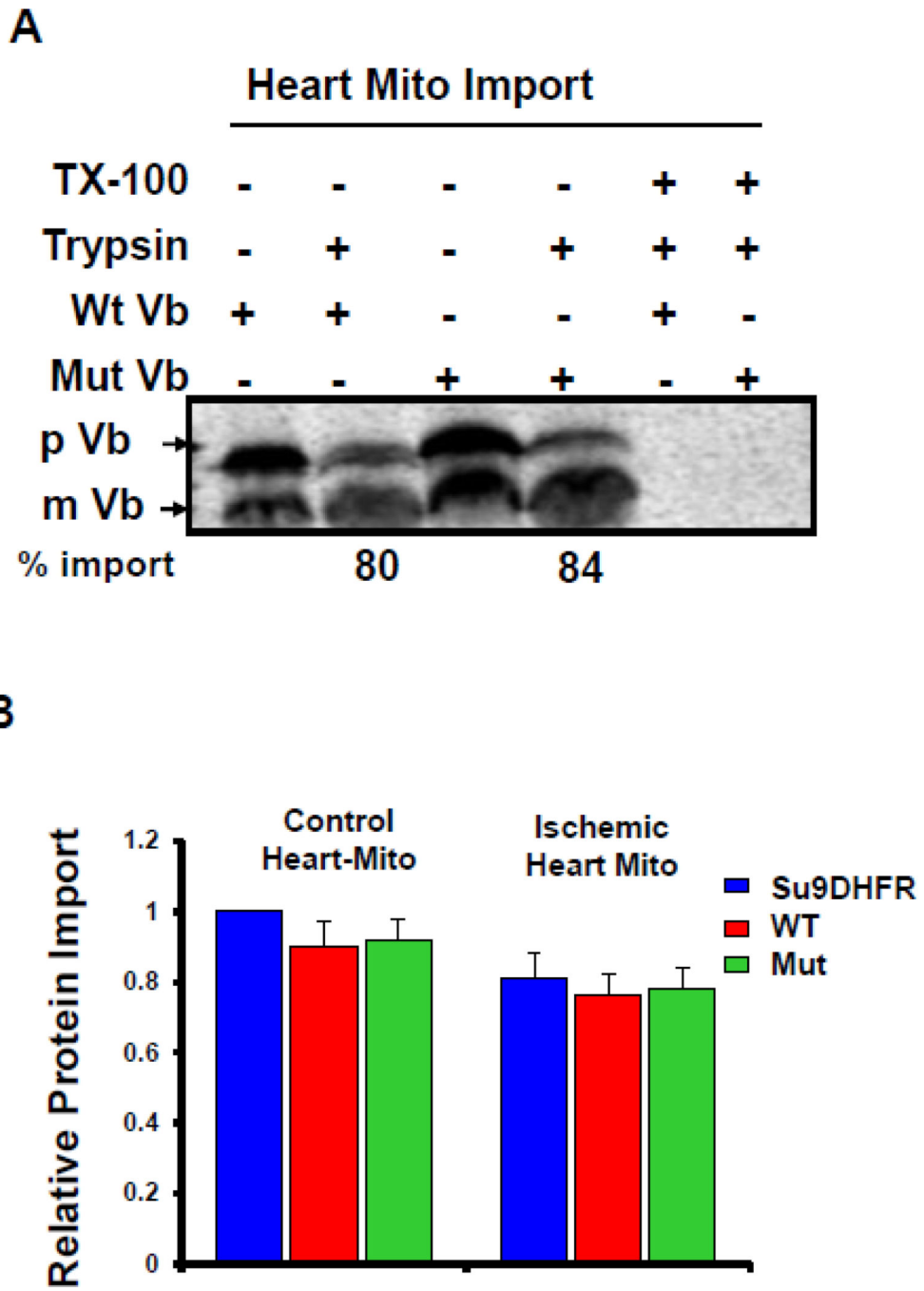

Figure 3.

Import efficiency of CcO subunits by mitochondria from control and ischemic hearts: Mitochondrial isolates from control and ischemic hearts were used for the import of ^{35}S labeled WT and S40A Vb proteins. A) Mitochondrial import of WT and S40A mutant Vb proteins. The fluorogram shows the positions of precursor (p) and mature (m) Vb proteins. B) shows the relative levels of import of WT and S40A mutant Vb proteins in comparison to SU9-DHFR protein used as a positive control by control heart and ischemic heart mitochondria. The values in B represent mean ±SD from three separate import assays.

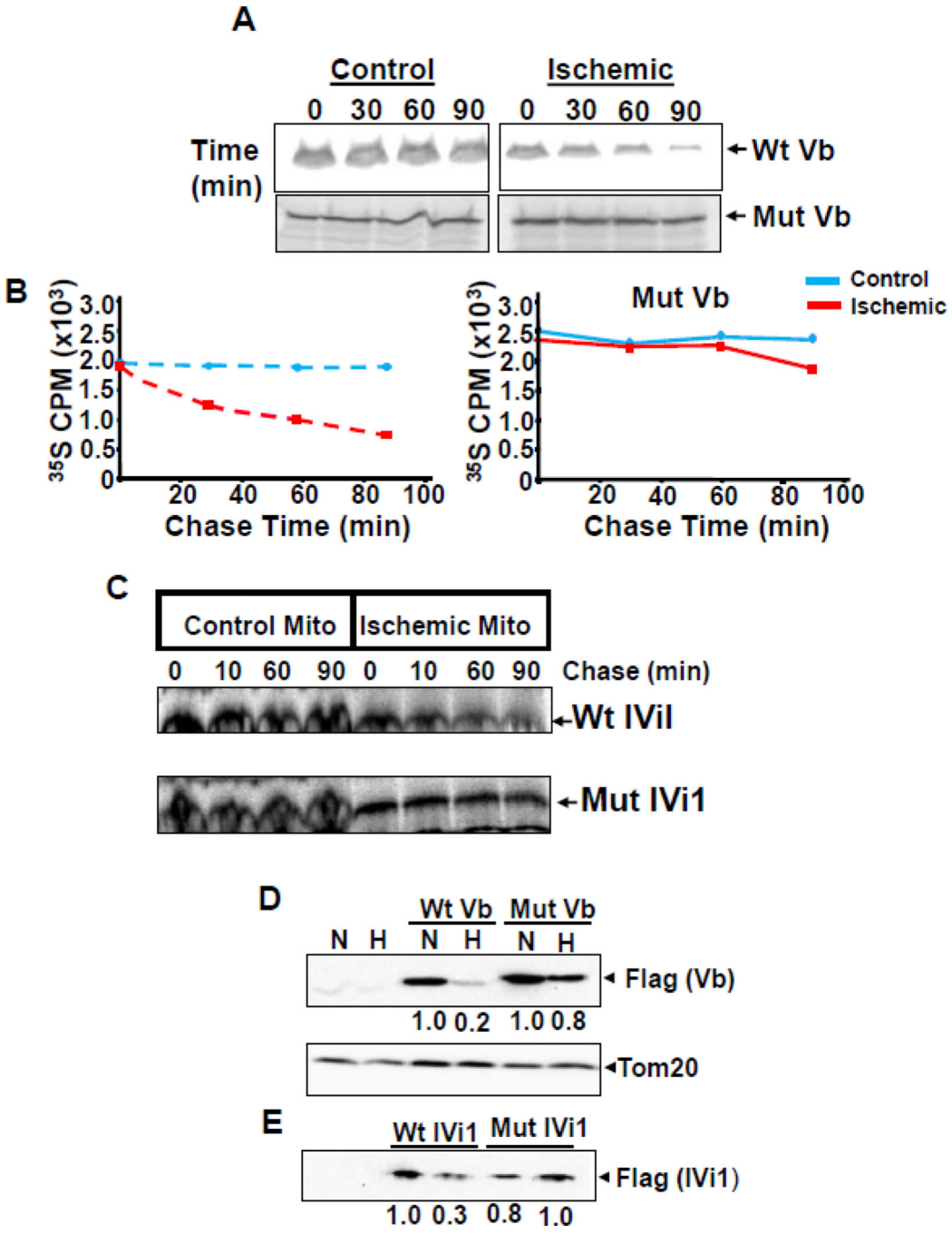

Figure 4.

Effects of ischemia/reperfusion injury and hypoxia on the turnover of CcO subunits. A) Relative turnover rates of ^{35}S labeled Vb proteins in mitochondria from control and ischemic hearts. The labeled WT and S40A Vb proteins were imported into isolated mitochondria and mitochondria were incubated in a chase medium for zero to 90 min as described in the Materials and Methods section. Following chase, mitochondrial proteins were resolved by SDS-polyacrylamide gel electrophoresis and subjected to fluorography. B) Relative levels of Vb proteins from in vitro incubated mitochondria in A were plotted against time of chase for

both WT and S40A mutant proteins. C) ^{35}S labeled CcO IVi1 (WT and T52A mutant) proteins were imported into mitochondria from control and ischemic hearts and subjected to in vitro chase as in Fig. A. Labeled proteins were resolved on SDS polyacrylamide gel as in A. D. Fluorograms in D show relative levels of Flag tagged WT Vb and S40A (Mut Vb) and also WT and mutant IViI proteins under normoxic and hypoxic conditions. RAW264.7 macrophages were transfected with empty vectors, WT or Mut cDNA constructs and cells were grown under normoxia or hypoxia for 12h. The mitochondrial proteins (30 μg each) were subjected to immunoblot analysis either with Flag antibody or TOM20 antibody for assessing loading levels. Values presented here represent mean of two independent experiments which did not deviate more than 15%.

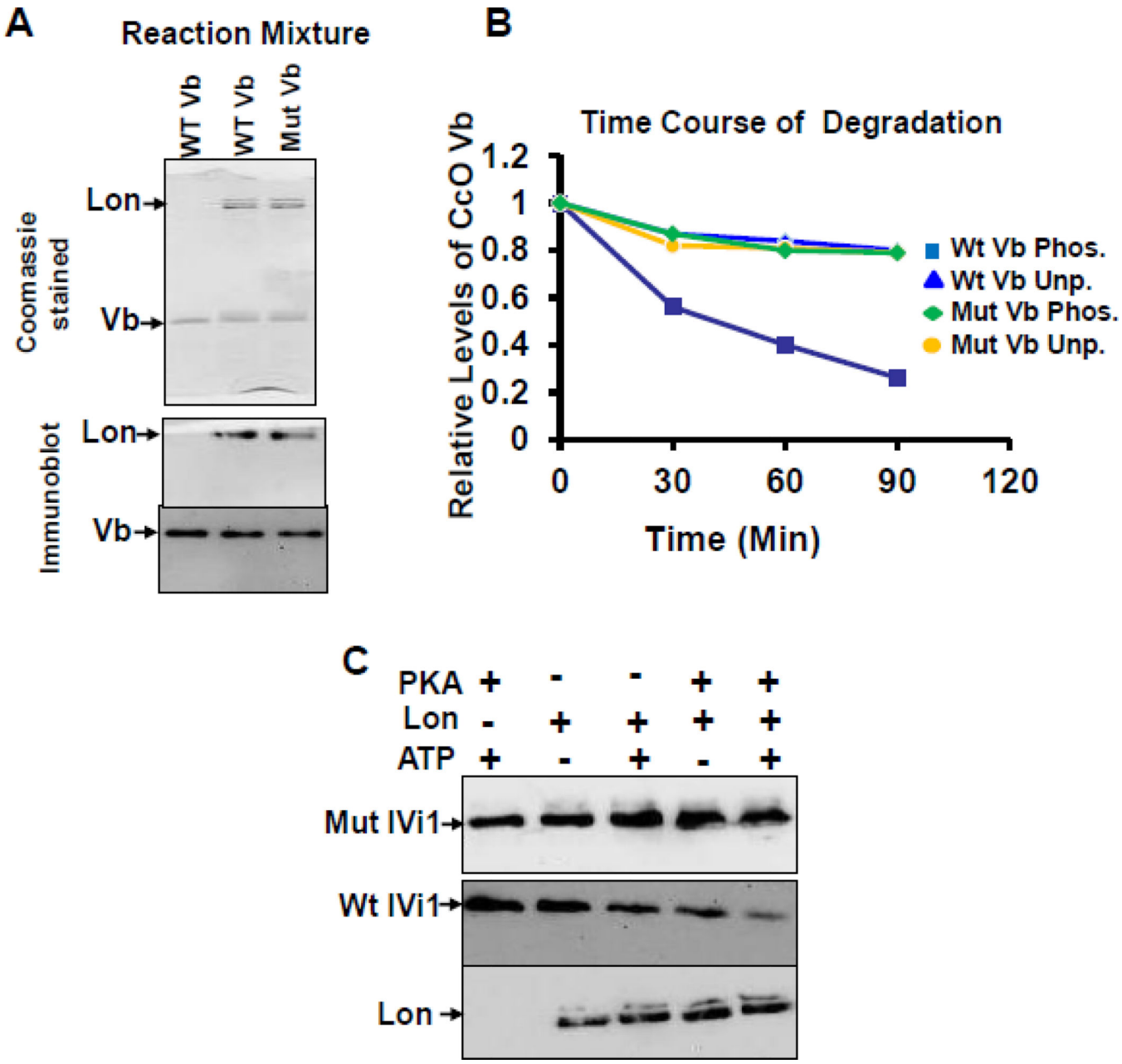

Fig. 5.

Lon mediated degradation of phosphorylated CcO Vb and IVi1 subunits *in vitro*: A) Recombinant purified wild type or phosphomutant S40A Vb protein along with purified Lon proteins were subjected to SDS-polyacrylamide gel electrophoresis. The upper panel represents a stained gel. The two lower panels represent immunoblots of parallel gels developed with Lon and Vb antibodies. B). Reactions with WT and mutant proteins preincubated with or without (Vb phos, and Vb unp, respectively) along with added Lon protein as in Fig. A were incubated for varying time points from zero to 90 min, resolved on SDS-PAGE and stained with coommassie Blue as in Fig A. The band intensities were determined and plotted against time as shown in B. The values represent mean of two

separate experiments. C). Similar reactions were run with WT and T52A mutant IVi1 with added Lon protein. The proteins were resolved on SDS-PAGE and subjected to immunoblot analysis with antibodies to IVi1 and Lon.

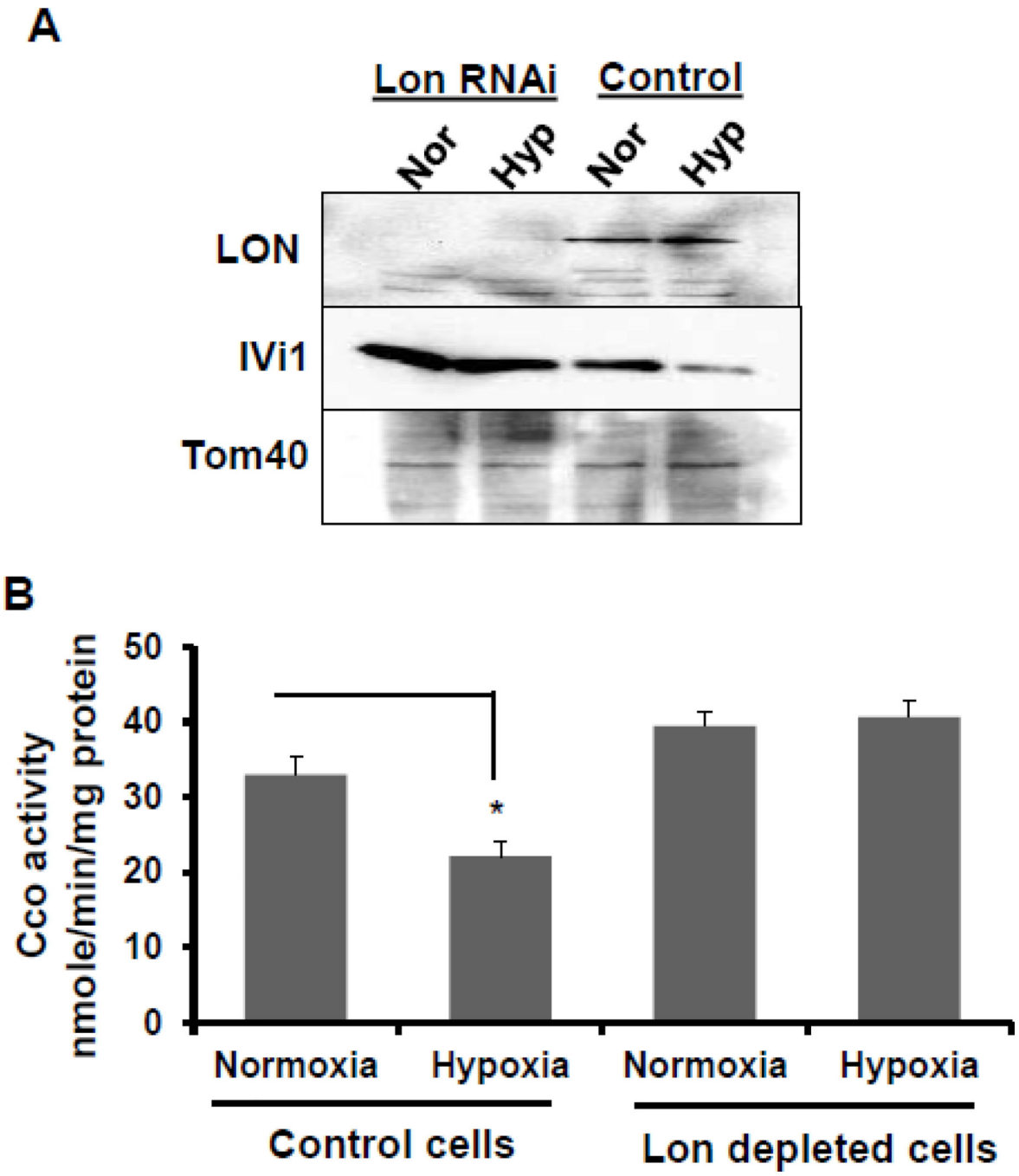

Figure 6.

Steady state levels of CcO subunit IVi1 in Lon depleted cells: Lon mRNA was silenced in human colon carcinoma cells by stable expression of antisense or non-specific oligonucleotides. Control and Lon depleted cells were grown for 6 days and subjected to normoxia (N) or hypoxia (H) for 12h. Isolated mitochondrial extracts were separated on SDS-PAGE and subjected to western blot analysis with indicated antibodies. B) Control or Lon Depleted (Lon RNAi) human colon carcinoma cell lines were subjected to hypoxic

Stress as above for 12h. Isolated mitochondrial fraction was used to measure the CcO activity as described in the methods. The values shown mean ± SD (n=3).

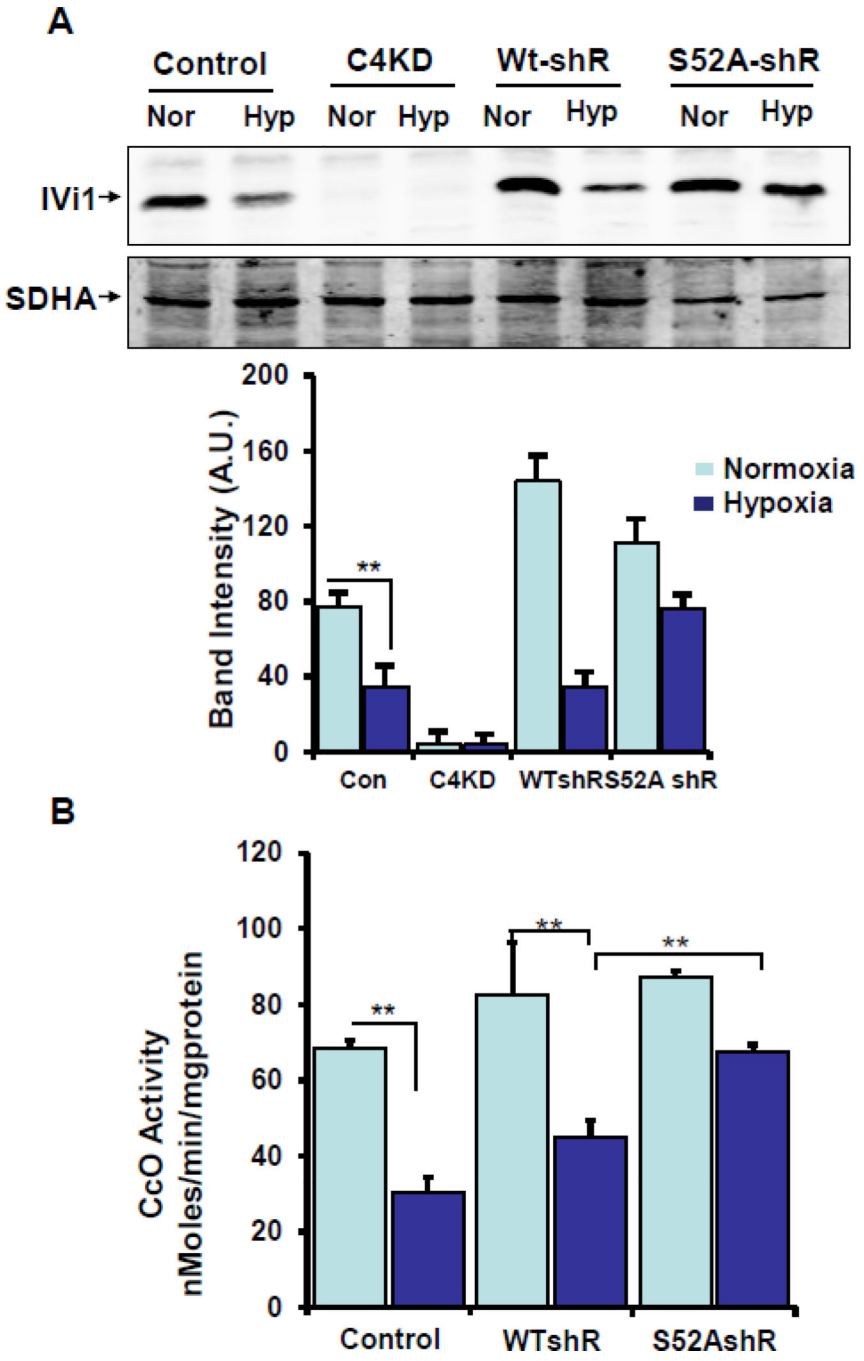

Figure 7.

Resistance of *in vivo* expressed S52A mutant of subunit IVi1 to hypoxic degradation. A) Stable cells lines expressing shRNA against CcO subunit IVi1 (C4KD) were transfected with degenerated wild type (Wt-shR) or phosphomutant (S52A–shR) CcO subunits as described in the methods. After 48h of transfection, ells were grown under normoxia or hypoxic (12 hrs at 0.2% oxygen) conditions and subjected to subcellular fractionation. Isolated mitochondrial extracts were separated on SDS-PAGE and probed with antibodies to CcOIVi1 and SDHA. Relative protein levels were shown in a histogram B) CcO activity was

measured spectrophotometrically by following oxidation of reduced cytochrome c with permeabilized mitochondria. The values represent mean ± SD (n=3).

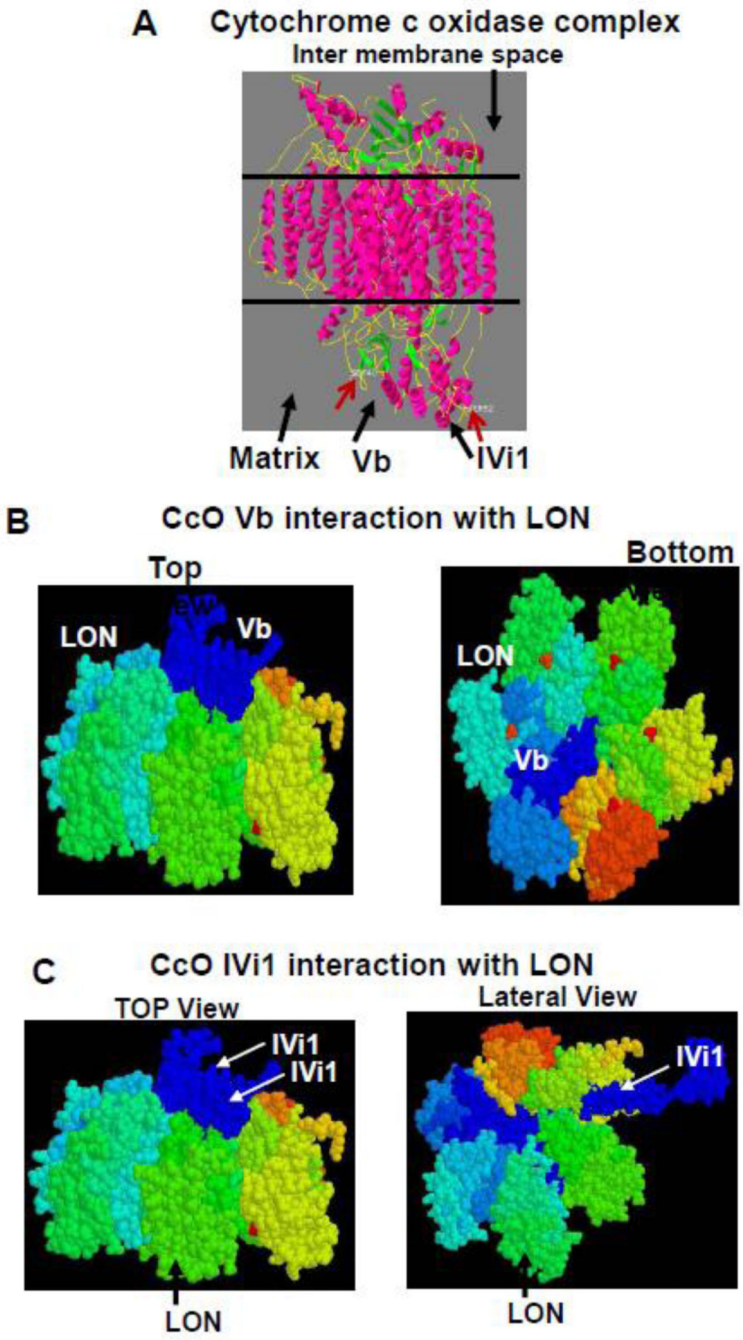

Fig 8.

Molecular modeling of CcO subunit phosphorylation sites and interaction with Lon protease. A) the 3D structure of CcO complex showing the phosphorylation sites S40 of Vb and T52 of IVi1 were based on the crystal structure coordinates reported before (35). B and C show the docking of subunits VB and IVi1, respectively on the hexameric structure of bacterial Lon reported before (36, 37).