Oxygen-sensitive mitochondrial accumulation of cystathionine β-synthase mediated by Lon protease

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Oxygen-sensitive accumulation and degradation, two opposite but intrinsically linked events, of heme proteins in mitochondria affect mitochondrial functions, including bioenergetics and oxygen-sensing processes. Cystathionine β-synthase (CBS) contains a prosthetic heme group and catalyzes the production of hydrogen sulfide in mammalian cells. Here we show that CBS proteins were present in liver mitochondria at a low level under normoxia conditions. Ischemia/hypoxia increased the accumulation of CBS proteins in mitochondria. The normalization of oxygen partial pressure accelerated the degradation of CBS proteins. Lon protease, a major degradation enzyme in mitochondrial matrix, recognized and degraded mitochondrial CBS by specifically targeting at the oxygenated heme group of CBS proteins. The accumulation of CBS in mitochondria increased hydrogen sulfide production, which prevented Ca^{2+}-mediated cytochrome *c* release from mitochondria and decreased reactive oxygen species generation. Mitochondrial accumulation of heme oxygenase-1, another heme protein, was also regulated by oxygen level and Lon protease in the same mechanism as for CBS. Our findings provide a fundamental and general mechanism for oxygen-sensitive regulation of mitochondrial functions by linking oxygenation level to the accumulation/degradation of mitochondrial heme proteins.

gasotransmitter | hepatocytes | mitochondrial swelling | signaling | transfection

Mitochondrial protein quality control system maintains homeostasis of mitochondria via regulated mitochondrial biogenesis and protein degradation (1, 2). Lon protease is a major protease in mitochondrial matrix in mammalian cells, being engaged in the degradation of proteins to prevent protein aggregation (2, 3). In doing so, Lon protease regulates many oxygen/ATP-dependent mitochondrial processes under physiological and pathophysiological conditions, such as DNA binding, chaperone activity, the assembly of respiratory complexes, and cellular aging and degeneration.

Mitochondrial heme-containing proteins are indispensable for normal mitochondrial function such as oxidative phosphorylation and biogenesis. The mechanisms by which Lon protease recognizes and regulates the degradation of mitochondrial heme proteins are unknown to date. Cystathionine β-synthase (CBS) is a nuclear encoding heme protein, playing a key role in homocysteine and cysteine metabolism and endogenous H_2S production (4–6). Deficiency of CBS as seen in autosomal recessively genetic disease causes homocystinuria, leading to dislocated optic lenses, skeletal disorders, mental retardation, and vascular disorders (4–6). As a gasotransmitter, H_2S regulates a wide variety of physiological events from vasorelaxation to glucose metabolism (4, 7, 8). We hypothesize that the presence and accumulation of CBS in mammalian mitochondria are regulated by an interaction of the heme group and oxygen molecule and this interaction is under the control of Lon protease. A better understanding of whether and how CBS in mammalian mitochondria is accumulated would help unmask the molecular mechanisms for oxygen-sensitive mitochondrial degradation of heme proteins. In this study, we demonstrated that ischemia/hypoxia caused the accumulation of CBS in mitochondria because Lon protease failed to efficiently degrade CBS. The oxygenation status of the heme group contained in CBS protein is the determining factor for CBS recognition and degradation by Lon protease in mitochondrial matrix. We also provide evidence that H_2S produced by mitochondrial CBS prevented Ca^{2+}-mediated cytochrome *c* (cyt *c*) release from mitochondria and decreased reactive oxygen species (ROS) generation. Our study, thus, unveils a fundamental mechanism for oxygen-sensitive and heme-dependent regulation of mitochondrial functions by linking oxygenation level to the accumulation/degradation of mitochondrial heme proteins.

Results

Accumulation of CBS in Mitochondria Under Normoxia and Hypoxia Conditions. Ischemia/reperfusion (I/R) damage was made to rat livers in vivo. Then, mitochondria were isolated from sham-operated, ischemia-damaged, and I/R-damaged rat livers. A basal level of CBS protein was detected in sham-operated mitochondrial fractions by Western blotting (Fig. 1 *A* and *B*). Liver ischemia increased CBS protein level in a time-dependent manner in mitochondria (Fig. 1 *A* and *B*), which was rapidly returned to basal level after reperfusion for 10 min (Fig. 1 *B* and *C*). Ischemia and I/R did not apparently affect the levels of CBS protein in cytosolic fractions or whole tissue homogenates of rat livers (Fig. S1). Thus, CBS accumulation in mitochondria is not due to the enhanced expression of CBS proteins. Cystathionine gamma-lyase (CSE) is another H_2S-generating enzyme, but it is not a heme-containing protein. In contrast to the mitochondrial localization of CBS, no CSE protein was detected in either sham-operated or ischemia-damaged liver mitochondrial fractions (Fig. 1 *A* and *B*). The absence of CSE in liver mitochondrial fractions but its presence in cytosolic fraction (Fig. S1) confirmed that our mitochondrial preparations were not contaminated by cytosolic fractions. Mitochondrial heat shock protein 70 (mtHsp70) is localized in mitochondrial matrix and functions as a translocation motor that chaperons cytosolic proteins into mitochondrial matrix in an ATP-dependent manner (9). CBS and mtHsp70 proteins were coimmunoprecipitated in mitochondrial fractions from both ischemic and sham-operated livers (Fig. 1 *D* and *E*). It appears that mtHsp70 was involved in mitochondrial CBS translocation under physiological conditions.

In cultured human hepatoma Hep3B cells, hypoxia increased mitochondrial level of CBS proteins (Fig. 2*A*). The crystal structure of human CBS shows that heme is bound to N-terminal loop (1–70 amino acid residues) and coordinated by residues Cys52 and His65, and the hemes are relatively surface-exposed in a dimeric CBS (10). We investigated the role of prosthetic heme

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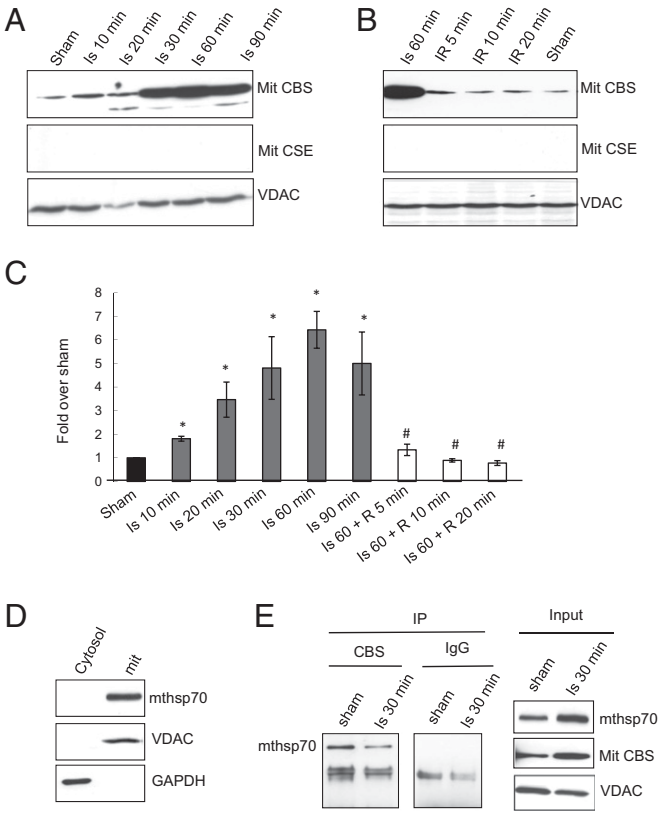

Fig. 1. Mitochondrial presence of CBS proteins under different conditions. (*A*) Mitochondrial presence CBS proteins (Western blot) in rat liver tissues undergoing hepatic ischemia or sham operation for different periods. (*B*) Mitochondrial presence of CBS proteins (Western blot) in rat liver tissues after 60 min hepatic ischemia followed by reperfusion for different times. (*C*) Ischemia- or ischemia/reperfusion-induced changes in mitochondrial CBS levels normalized against voltage-dependent anion channel (VDAC) levels, relative to that in the absence of hepatic ischemia damage (sham) (*$P < 0.05$ versus sham, $^{\#}P < 0.05$ versus Is60 min). (*D*) Distribution of mitochondrial heat shock protein 70 (mitHsp70) in cytosolic and mitochondrial fractions detected with Western blotting. (*E*) Mitochondrial fractions were prepared from rat livers with sham operation or after 30 min of liver ischemia. Immunoprecipitation was performed using anti-CBS antibody and analyzed with Western blotting using both anti-mitHsp70 and anti-CBS antibody. CBS and mitHsp70 in mitochondria lysis, as input, were also detected with Western blotting. Mit, mitochondria; Is, ischemia; R, reperfusion; IR, ischemia followed by reperfusion. The blots in *A*–*D* were striped and reprobed with anti-VDAC antibody as a mitochondrial marker or with anti-GAPDH antibody as a cytosol marker. $n = 3$–4 for each group.

in hypoxia-mediated accumulation of CBS by genetically altering the heme-binding sequence of CBS proteins. We prepared plasmids encoding wild type (WT) and mutated [C52A, H65R, and the whole heme-binding domain deletion (Δ1–70)] human *CBS* gene (Fig. S2). HEK293 cells were transiently transfected with these expression plasmids, and the ectopic expression of a given gene was confirmed by Western blot assay with a myc-tag antibody. In comparison with the expression levels of WT-CBS proteins, Δ1–70 CBS mutant was significantly accumulated in the mitochondria, whereas a lower level of Δ1–70 CBS mutant was detected in the cytosol under normoxia condition (Fig. 2*B*). Specifically mutating C52A and H65R also caused accumulation of CBS mutants in mitochondria under normoxia. Because CBS mutants and WT CBS have the same expression levels in cytosol, mitochondrial accumulation of CBS mutants could not be due to the overexpression of mutated *CBS* (Fig. 2*B*). We further established HEK293 cell lines stably expressing WT and mutated (Δ1–70 and H65R) *CBS* genes (Fig. S3 *A* and *B*). Consistent with the results of transient transfection of *CBS* mutants, pronounced mitochondrial accumulation of Δ1–70 and H65R mutated CBS proteins was observed in these stably transfected cell lines (Fig. S3*C*). These observations indicate that the accumulation of CBS mutants in mitochondria might be due to the lack of heme group. Although WT CBS proteins were accumulated in mitochondria in response to hypoxia, hypoxia failed to induce mitochondrial accumulation of the mutated CBS proteins (Fig. 2 *C*–*E*). Together, our data suggest that the hypoxia-mediated accumulation of CBS protein depends on the presence of the prosthetic heme. Hypoxia leads to deoxygenation-related conformational change of the heme so that CBS cannot be recognized and degraded by the heme-targeted enzyme(s).

$CoCl_2$ and desferrioxamine (DFX) have been frequently used to mimic hypoxia under normoxic condition. These two hypoxia-mimetic agents also enhanced mitochondrial CBS protein levels under normoxic condition (Fig. 2 *F* and *G*). $CoCl_2$ and DFX may cause a conformational change of CBS through substituting for or chelating ferrous irons in the heme group (11), rendering the loss of oxygen-binding ability of the heme. Therefore, these hypoxia-mimetic agents could stabilize mitochondrial CBS protein against degradation by locking CBS at a deoxygenated status.

To examine whether mitochondrial CBS protein level under normoxic condition depends on the dynamics of protein degradation, Hep3B cells were treated with MG132, an inhibitor of Lon protease (12, 13). MG132 alone elevated mitochondrial level of CBS proteins (Fig. 3*A*). Knockdown of Lon protease

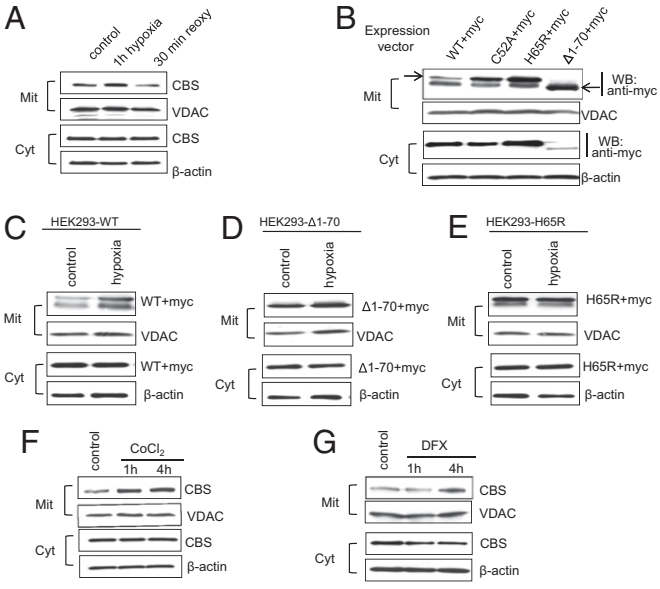

Fig. 2. Heme-based oxygen-sensitive regulation of mitochondrial CBS accumulation. (*A*) Distribution of CBS in cytosolic and mitochondrial fractions (Western blotting) after Hep3B cells were incubated under normoxia, hypoxia, or reoxygenation. (*B*) Distribution of wide-type (WT) CBS or mutated CBS proteins in cytosolic and mitochondrial fractions, respectively, after HEK293 cells were transiently transfected with the indicated expression vectors for 24 h. An anti-myc antibody was used in Western blotting. (*C*–*E*) HEK293 cells, stably transfected with WT (*C*), Δ1–70 (*D*), or H65R (*E*) CBS, were incubated under hypoxia or normoxia condition for 4 h before cytosolic and mitochondrial fractionation. Western blotting was performed using anti-myc antibody. (*F*) Distribution of CBS in cytosolic and mitochondrial fractions (Western blot) after Hep3B cells treated with or without 200 μM $CoCl_2$ for the indicated times. (*G*) Experiments were performed as in *F* except that cells were treated with desferrioxamine (100 μM). The blots from *A*–*G* were striped and reprobed with anti-VDAC antibody as a mitochondrial internal control or with anti–β-actin antibody as a cytosol internal control. NS, nonspecific bands. Mit, mitochondria. Cyt, cytosol. reoxy, reoxygenation. $n = 3$ for each group.

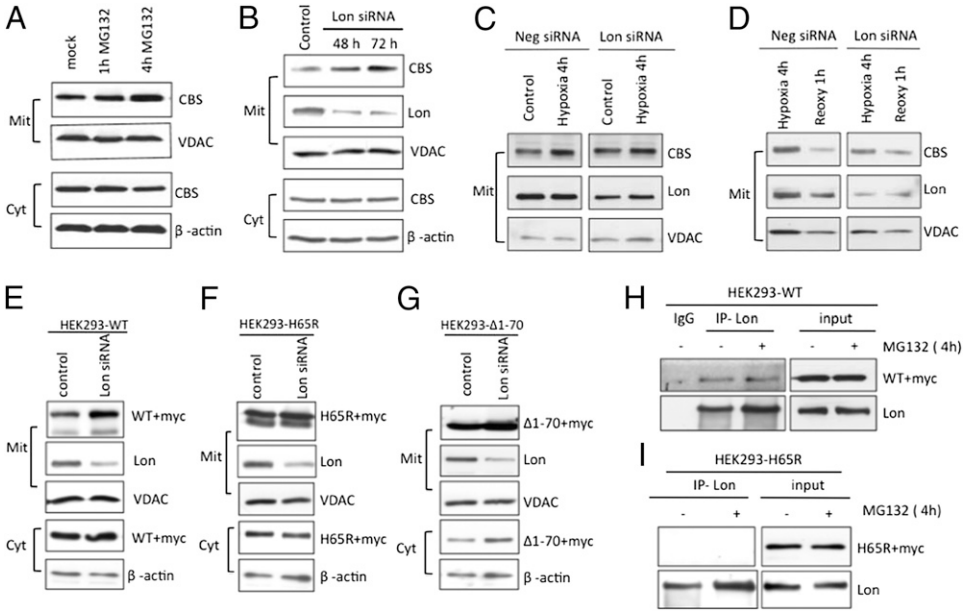

Fig. 3. Heme-based oxygen-sensitive regulation of mitochondrial CBS by Lon protease. (*A*) The abundance of CBS in cytosolic and mitochondrial fractions (Western blot) after Hep3B cells treated with or without MG132 (20 μM) for 1 h and 4 h. (*B*) CBS abundances in cytosolic and mitochondrial fractions after Hep3B cells were treated with control siRNA or siRNA-*Lon protease* for different times. (*C*) Hep3B cells were treated with control siRNA or siRNA-*Lon protease* for 48 h followed by normoxia or hypoxia for the indicated times. Mitochondrial fractions were prepared for Western blotting. (*D*) Experiments were performed as in *C* with the emphases on hypoxia-reoxygenation treatment. The blots from *A*–*D* were striped and reprobed with anti-VDAC antibody as a mitochondrial internal control or with anti–β-actin as a cytosol internal control. (*E*) HEK293-WT cells were treated with control siRNA or siRNA-*Lon protease* for 72 h and then Western blotting analyses were done. (*F*) HEK293-H65R CBS cells were treated the same way as in *E*. (*G*) HEK293-Δ1–70 CBS cells were treated the same way as in *E*. (*H*) HEK293-WT CBS cells were treated with or without MG132 (20 μM) for 4 h before lysis and coimmunoprecipitation with anti-Lon antibody. CBS in the immunoprecipitate was detected by Western blotting with anti-myc antibody. $n = 3$ for A-H. (*I*) Experiments were performed as in *H* on HEK293-H65R CBS cells. The blots from *A*–*G* were striped and reprobed with anti-VDAC antibody as a mitochondrial internal control or with anti–β-actin antibody as a cytosol internal control.

with specific siRNA significantly elevated mitochondrial CBS protein level in Hep3B cells, but had no effect on cytosolic CBS protein level (Fig. 3*B*). These results indicate that CBS is a physiological substrate of Lon protease in mitochondria.

Heme-containing proteins have been suggested as oxygen sensors (11). Increased CBS protein accumulation in mitochondria during hypoxia represents an oxygen-responsive event, which may transmit oxygen signal to alter mitochondrial H_2S production. We next tested whether hypoxia-regulated CBS turnover in mitochondria is mediated by Lon protease. After siRNA suppression of Lon protease expression in Hep3B cells, hypoxia-induced mitochondrial accumulation of CBS proteins was largely eliminated (Fig. 3*C*). Reoxygenation-induced normalization of mitochondrial CBS protein levels was also significantly reduced after siRNA knockdown of Lon protease (Fig. 3*D*). Knockdown of Lon protease conspicuously increased accumulation of WT CBS protein in mitochondria (Fig. 3*E*), but not H65R-CBS and Δ1–70-CBS mutants in HEK-293 cells (Fig. 3 *F* and *G*). Coimmunoprecipitation assays revealed that WT CBS protein (Fig. 3*H*), but not H65R-CBS mutant (Fig. 3*I*), was recruited to Lon protease.

Mitochondrial Targeting Sequence in C-Terminal Regulatory Region of CBS Proteins. Most mitochondrion-oriented proteins contain a cleavable presequence at their *N* termini. Some mitochondrial imported proteins have uncleavable internal targeting signals instead of the presequence (14). We searched for mitochondrial targeting sequence of CBS responsible for mitochondrial import. To this end, expression vectors of WT or various deletion mutants of *CBS* were prepared (Fig. S2) and transfected into HEK293 cells. Two N-terminal truncated mutants of CBS (Δ1–337 and Δ1–380) were detected in mitochondria (Fig. 4*A*), indicating that the mitochondrial targeting signals do not reside in 1–380 amino acid sequence of the N terminus. C-terminal regulatory region of CBS contains two tandem repeats of domain previously defined as CBSD1 and CBSD2 (Fig. S2) (15). No mitochondrial localization of Δ468–551 (lack of CBSD2 motif) or Δ416–551 (lack of both CBSD1 and CBSD2 motifs) was detected, although these mutants were detected in the cytosol (Fig. 4 *A* and *B*). On the other hand, Δ411–470 (lack of CBSD1 motif) was detected in the mitochondria (Fig. S4). As such, the internal targeting signals may reside within C-terminal regulatory domain of CBS protein, most likely at CBSD2 motif. Through binding and delivering proteins to translocation machinery on mitochondrial surface, cytosolic Hsp70 and Hsp 90 have been implicated in the import process of proteins (16). Deletion of C-terminal regulatory domain of CBS protein abolished interaction between Hsp70, but not Hsp90, and mutated CBS protein (Fig. 4*C*).

Mitochondria-Produced H_2S and Mitochondrial Release of Cyt *c* and Production of ROS. Mitochondrial accumulation of CBS affects mitochondrial function. Hepatic ischemia caused no change in either CSE or CBS expression or H_2S production in whole liver tissues (Fig. 5*A*). A significant increase in H_2S production rate was detected in mitochondrial fractions isolated from ischemic liver tissues compared with that isolated from sham-operated liver tissues (Fig. 5*B*). Amino-oxyacetate (AOA), a CBS inhibitor, blocked mitochondrial H_2S generation (Fig. 5*B*). We next examined whether H_2S could prevent Ca^{2+}-induced cyt *c* release from mitochondria and mitochondrial swelling. Treatment with NaHS, a H_2S donor, before Ca^{2+} addition completely abolished Ca^{2+}-induced release of cyt *c* from incubated liver cell mitochondria (Fig. 6*A*). The inhibitors (glibenclaminde and 5-HT) of ATP sensitive K^+ (K_{ATP}) channels (17) failed to block this effect of NaHS (Fig. 6*B*). Consistent with the blockage of Ca^{2+}-induced cyt *c* release, NaHS also inhibited Ca^{2+}-induced mitochondrial swelling (Fig. 6 *C* and *D*).

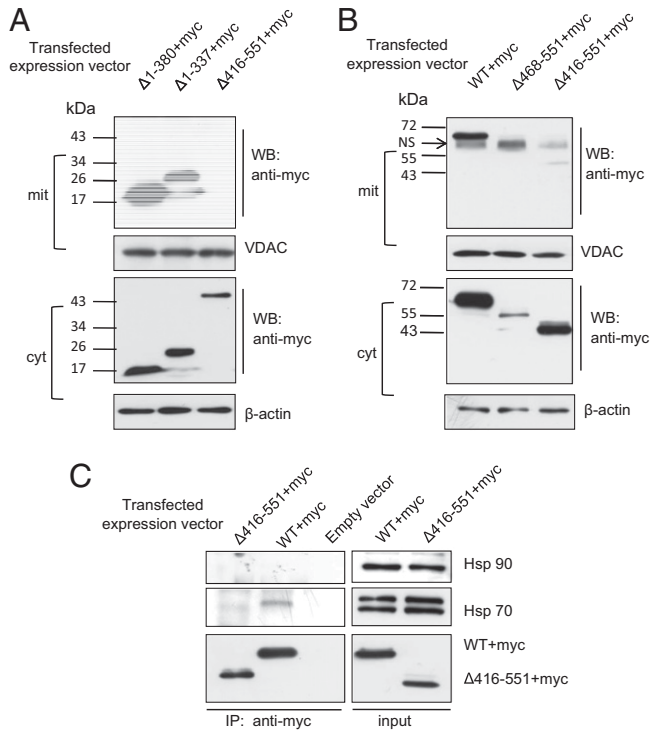

Fig. 4. Mitochondrial targeting signal(s) within the C terminus of CBS. (*A*) Distribution of truncated CBS proteins in cytosolic and mitochondrial fractions (Western blot) after HEK293 cells were transiently transfected with the indicated expression vectors, respectively, for 24 h ($n = 3$). (*B*) Experiments were carried out as in *A* except that different expression vectors were used as indicated ($n = 3$). (*C*) The interactions of CBS with Hsp70 and Hsp90 were determined by coimmunoprecipitation assay. Cells were transfected with empty control vector or vector encoding WT or Δ416–551*CBS*. NS, nonspecific bands.

H_2S has been reported to exert its effect by scavenging ROS (18, 19), and mitochondria are the major source of ROS production (20). Knockdown of CBS expression with specific siRNA significantly increased production of ROS in Hep3B under hypoxia condition, but did not affect ROS production under normoxia condition (Fig. 6*E*). Sixty minutes after the initiation of reoxygenation, ROS levels were not different with or without CBS knockdown (Fig. 6*E*). This result is in line with our observation that the accumulation of CBS in mitochondria is rapidly degraded upon reoxygenation.

Lon Protease Mediated Mitochondrial Accumulation of Other Heme Proteins. To test whether the oxygen-sensitive and Lon-mediated heme protein accumulation in mitochondria is a general phenomenon, we also tested another heme protein, heme oxygenase-1 (HO-1) (21). HO-1 proteins were detected in the mitochondria of Hep3B cells. After exposure to hypoxia, HO-1 proteins were accumulated in mitochondria (Fig. S5*A*). siRNA knock-down of Lon protease resulted in mitochondrial accumulation of HO-1 under normoxia condition (Fig. S5*B*).

Discussion

Recognition of oxidatively modified proteins and their subsequent removal prevent mitochondrial protein accumulation, maintain mitochondrial viability, and delay the aging process (1, 2). Lon protease is highly conserved from prokaryotes to the mitochondria of eukaryotes. A previous study suggested that Lon protease degrades preferentially oxidatively damaged proteins in mitochondrial matrix (3). Lon protease efficiently degrades a stably folded protein by recognition of the targeting signal (22). A recent study indicated that the degradation of delta-aminolevulinic acid synthase involved the heme structure and the activation of Lon protease (23). In previous studies, CBS was detected in the cytosol but not in mitochondria of rat liver cells (24). Our present study demonstrates that the turnover of CBS, a heme protein, in liver mitochondria is constitutively regulated by Lon protease in a heme-based and oxygen-sensitive fashion. Heme groups are relatively surface-exposed in CBS proteins (10). More specifically, the heme group binds to H65 and C52 of CBS protein noncovalently (25). Our site mutagenesis results further demonstrated that C52 and H65 are important for oxygen sensing and for Lon protease to access and recognize heme-containing motif of CBS proteins. With mutated C52 or H65, the heme might move away from surface to inner space of CBS protein with several hydrophonic amino acids around. Consequently, Lon protease cannot degrade these forms of CBS as no heme is on the surface. The same structural recognition mechanism applies to HO-1 in which the heme group is also surface exposed with one side of heme binding to histidine and the other side to other ligand(s) via weak hydrogen bond (26). Under normoxic condition, oxygen binds to heme and causes a conformational change with which heme becomes available for Lon protease recognition, leading to the degradation of CBS proteins. Ischemia leads to the accumulation of CBS in liver mitochondria as the heme groups of CBS proteins are deoxygenated and could not be recognized by Lon protease (Fig. 7). The normalization of oxygen partial pressure after reperfusion accelerates the degradation of CBS proteins.

Predicted by the sequence analyzing programs (PSORT II and MitoProt II), CBS does not contain a cleavable N-terminal signal to direct it to mitochondrial matrix. However, CBS proteins were detected in our study in both cytosolic and mitochondrial fractions of hepatocytes. Thus, CBS may contain internal signals that direct CBS to mitochondria. As shown in our study, the C-terminal regulatory region of CBS contains putative internal targeting signals, and this region is required for CBS interacting with cytosolic Hsp70. Cytosolic Hsp70 has been demonstrated to play an important role in delivering mitochondrial targeting proteins to import receptor on the surface of mitochondria (16, 27).

Previous studies have shown that exogenously administered H_2S significantly ameliorated myocardial and hepatic I/R injury (28, 29). The protective effect of exogenous H_2S is attributed to the preservation of mitochondrial function (28, 29), but how this

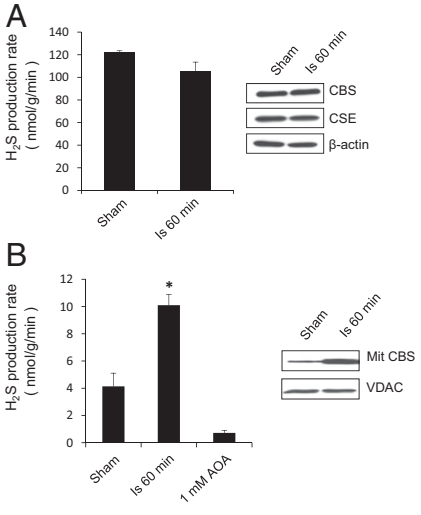

Fig. 5. H_2S production rates under different conditions. (*A*) Measurement of H_2S production rate in liver tissues collected after 60 min ischemia (Is 60 min) or sham operation. (*B*) Measurement of H_2S production rate in mitochondria fraction from sham-operated and ischemic (Is 60 min) liver tissues (*$P < 0.05$ versus other groups, $n = 6$ for *A* and *B*). The expression levels of CBS and CSE proteins under different conditions were shown as *Insets*.

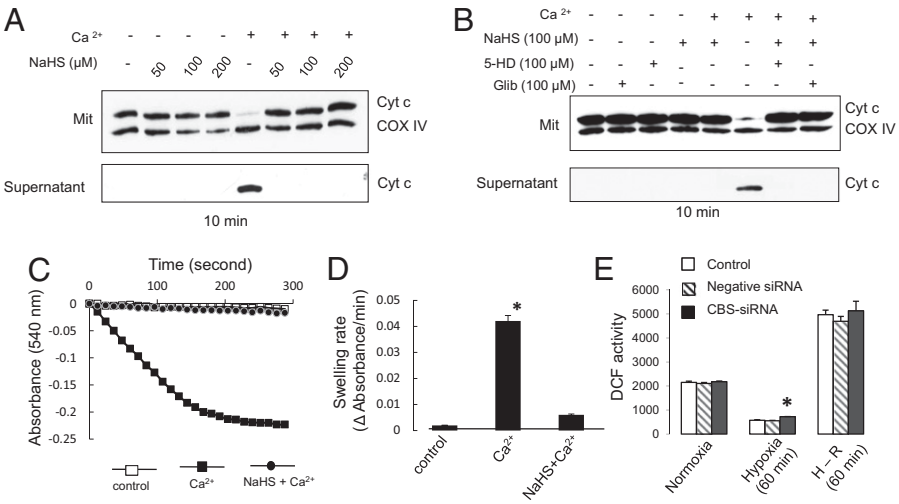

Fig. 6. Effects of H_2S and CBS on Ca^{2+}-induced cyt *c* release and ROS production. (*A*) The presence of cyt *c* in the mitochondrial fraction and incubated supernatant (Western blotting) after mitochondria were incubated with Ca^{2+} (50 nM per mg of mitochondrial protein), treated with NaHS before Ca^{2+} addition, or treatments with different concentrations of NaHS for 10 min. The blots were striped and reprobed with an anti-complex IV cyt *c* oxidase (COX IV) antibody as a mitochondrial inner membrane marker. (*B*) Experiments were performed as in *A* except that mitochondria were treated differently. (*C*) Incubated mitochondria were pretreated with or without NaHS (100 μM) followed by addition of Ca^{2+} (50 nM per mg of mitochondrial protein). Mitochondrial swelling was measured by absorbance at 540 nm for the indicated time. (*D*) The rate of swelling represents average decrease in absorbance within 5 min (*$P < 0.05$ versus NaHS+Ca^{2+}). (*E*) Hep3B cells were treated with control siRNA or siRNA-*CBS* for 48 h followed by normoxia, hypoxia treatment, or hypoxia-reoxygenation treatment for 1 h. Intracellular ROS was measured as DCF fluorescent intensities (*$P < 0.01$ versus control siRNA group under hypoxia). AOA, amino-oxyacetate; Glib, glibenclaminde; H-R, reoxygenation after hypoxia; I, ischemia. $n = 3–5$ for each group.

"preservation" is achieved by endogenous H_2S has been unclear. One of the mechanisms could be related to increased ATP production by mitochondrial produced H_2S in the presence of hypoxia (30). Increased cytosolic Ca^{2+} is a key event in the pathological process of I/R injury because Ca^{2+} can induce the opening of mitochondrial permeability transition pore and lead to mitochondrial swelling and the release of cyt *c* from mitochondria, initiating cell apoptosis and necrosis (31, 32). A rapid increase in hepatocyte Ca^{2+} was detected after the onset of reperfusion, but not during the ischemic phase (33). In the present study, we show that mitochondrial CBS accumulation during liver ischemia is associated with increased production of H_2S in mitochondria, and H_2S inhibits Ca^{2+}-induced cyt *c* release and mitochondrial swelling in vitro. Furthermore, we show that mitochondrial CBS accumulation during hypoxia decreases ROS production. These findings suggest that the accumulation of CBS and increased production of H_2S in mitochondria may be required for protecting mitochondria from I/R injury. A prolonged mitochondrial H_2S production, however, may damage mitochondrial functions. Therefore, a rapid degradation of accumulated CBS followed by reperfusion provides an acute mechanism to terminate mitochondrial production of H_2S once oxygen partial pressure in mitochondria returns to the physiological level.

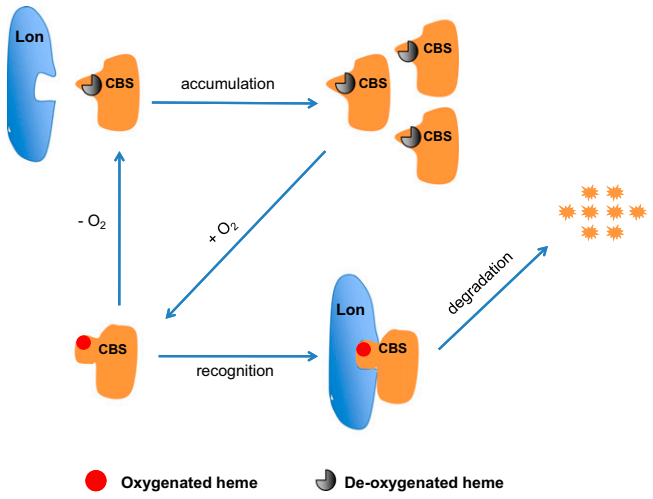

Fig. 7. The mechanisms for Lon protease-mediated mitochondrial CBS turnover. Lon protease degrades mitochondrial CBS through recognizing oxygenated heme of CBS. Ischemia/hypoxia leads to accumulation of CBS proteins in mitochondria because Lon protease fails to recognize CBS with deoxygenated heme.

Although the present study did not detect mitochondrial translocation of CSE in liver cells, our previous study reported a Ca^{2+}-induced CSE translocation into mitochondria of vascular smooth muscle cells (VSMCs) (30). The only H_2S-generating enzyme expressed in VSMCs is CSE but both CBS and CSE are involved in H_2S production in liver cells (6, 34). Furthermore, CSE protein does not reside in VSMC mitochondria under normal conditions whereas CBS protein is physiologically found in hepatocyte mitochondria. Thus, CSE and CBS function differently in VSMCs and liver cells. Different mechanisms also exist to regulate mitochondrial translocation of CBS and CSE in different types of cells.

In conclusion, oxygenation status of the heme group contained in CBS is the determining factor for the recognition and degradation of mitochondrial CBS by Lon protease. Lon protease may also regulate the turnover of other heme-containing proteins in mitochondria, such as HO-1, through recognizing a heme-dependent motif. Liver ischemia leads to the accumulation of CBS in mitochondria and increased H_2S production, which prevented Ca^{2+}-mediated cyt *c* release from mitochondria and hypoxia-induced mitochondrial ROS production. Thus, our findings stipulate a fundamental mechanism for oxygen-sensitive regulation of mitochondrial function by linking oxygenation level to the degradation of mitochondrial heme proteins.

Materials and Methods

Animal and Reagents. Male Sprague–Dawley rats (8–12 wk) were purchased from Jackson Laboratories. All animal experimental procedures were in compliance with the Guide for the Care and Use of Laboratory Animals

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published by the US National Institutes of Health and approved by the Animal Care Committee of Lakehead University. Animals were maintained on standard rodent chow and had free access to food and water. All reagents were purchased from Sigma unless otherwise indicated. The solution of NaHS was freshly prepared before use (6).

Liver Ischemia/Reperfusion. The surgical procedures for liver ischemia/reperfusion have been described (22). Briefly, laparotomy was performed on rats under anesthesia, and liver was exposed following midline incision. A microaneurysm clamp was used to the hepatic artery and portal vein to induce ischemia of the liver. At the end of the ischemic period, vascular clamp was removed and the liver was reperfused. Rats were subjected to 10, 20, 30, 60, and 90 min of liver ischemia or 5, 10, 20, and 30 min of reperfusion after 60 min of ischemia, respectively. After surgery, liver tissue was collected for various experimental assays.

Cell Culture and Hypoxic Treatment. HEK293 and Hep3B cells were obtained from ATCC and cultured in DMEM supplemented with 10% (vol/vol) FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in an atmosphere of 5% CO_2. For hypoxic treatment, cells were exposed to hypoxia in a chamber (STEMCELL Technologies) at 37 °C with a gas mixture of 5% (vol/vol) CO_2 and 95% (vol/vol) N_2.

Measurement of oxygen partial pressure (pO_2) in the cell culture medium was carried out using an electronic blood-gas analyzer (GEM Premier 3000, Instrumentation Laboratory). The pO_2 was 182.5 ± 2.6 mmHg under normoxic condition and 53.8 ± 4.84 mmHg under hypoxic condition. After desferrioxamine and $CoCl_2$ treatments, pO_2 reamained at 184 ± 1.47 mmHg and 175.75 ± 3.72 mmHg, respectively. $n = 4$ for each condition. Mitochondrial swelling was accessed by absorbance changes at 540 nm (35).

Preparation of Mitochondrial and Cytosolic Fractions and Measurement of H_2S Production. Mitochondria were isolated according to a protocol described (30, 36). The mitochondrial and cytosolic fractions were saved at −80 °C for future analysis. H_2S production by different cellular fractions was measured as described (6).

Western Blotting and Coimmunoprecipitation. Liver tissues, cultured cells, or isolated mitochondria were lysed and analyzed by Western blot. The primary antibodies used were: mouse monoclonal anti-CBS (1:1,000), anti-GAPDH (1:2,000), rabbit polyclonal anti-LONP1 (anti-Lon) (1:500) (NOVUS Biologicals); mouse monoclonal anti-cyt *c* (1:500) (BD Biosciences); mouse monoclonal anti-myc (1:5,000) (Invitrogen); mouse monoclonal anti-COX IV (1:1,000), rabbit polyclonal anti-voltage-dependent anion channel (VDAC) (1:1,000) (Cell Signaling); rabbit polyclonal anti-Grp75 (anti-mtHsp70) (1:1,000), anti-Hsp70 (1:1,000), anti-Hsp90 (1:1,000; Stressgen); rabbit polyclonal anti-CSE (1:500) (6); rabbit polyclonal anti–HO-1 (1:1,000; Cell Signaling); and mouse monoclonal anti–β-actin (1:5,000).

For coimmunoprecipitation assay, 1–2 mg of protein extracts were incubated with 2–3 μg of anti-CBS or anti-Lon protease antibody for 4 h at 4 °C followed by incubation with protein G or A Sepharose (GE Healthcare) for 1 h at 4 °C. The beads were washed three times with lysis buffer, and bound proteins were eluted by boiling for 5 min with 2× SDS loading buffer and analyzed by Western blotting.

Statistical Analysis. All data are expressed as means ± SE and represent at least three independent experiments. Statistical comparisons were made using Student *t* test or one-way analysis of variance followed by a post hoc analysis (Tukey test) where applicable, and $P < 0.05$ was considered statistically significant.

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