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S-sulfhydration of ATP synthase by hydrogen sulfide stimulates mitochondrial bioenergetics

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Abstract

Mammalian cells can utilize hydrogen sulfide (H_2S) to support mitochondrial respiration. The aim of our study was to explore the potential role of *S*-sulfhydration (a H_2S-induced posttranslational modification, also known as S-persulfidation) of the mitochondrial inner membrane protein ATP synthase (F1F0 ATP synthase/Complex V) in the regulation of mitochondrial bioenergetics. Using a biotin switch assay, we have detected *S*-sulfhydration of the α subunit (ATP5A1) of ATP synthase in response to exposure to H_2S *in vitro*. The H_2S generator compound NaHS induced *S*-sulfhydration of ATP5A1 in HepG2 and HEK293 cell lysates in a concentration-dependent manner (50–300 μM). The activity of immunocaptured mitochondrial ATP synthase enzyme isolated from HepG2 and HEK293 cells was stimulated by NaHS at low concentrations (10–100 nM). Site-directed mutagenesis of ATP5A1 in HEK293 cells demonstrated that cysteine residues at positions 244 and 294 are subject to *S*-sulfhydration. The double mutant ATP synthase protein (C244S/C294S) showed a significantly reduced enzyme activity compared to control and the single-cysteine-mutated recombinant proteins (C244S or C294S). To determine whether endogenous H_2S plays a role in the basal *S*-sulfhydration of ATP synthase *in vivo*, we compared liver tissues harvested from wild-type mice and mice deficient in cystathionine-gamma-lyase (CSE, one of the three principal mammalian H_2S-producing enzymes). Significantly reduced *S*-sulfhydration of ATP5A1 was observed in liver homogenates of $CSE^{-/-}$ mice, compared to wild-type mice,

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Competing interests

The authors declare no conflicts of interest in relationship to this study.

Authors' contributions

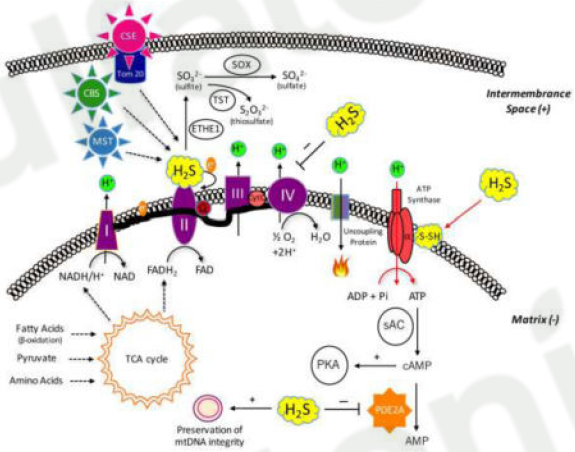
Katalin Módis: conduction of experiments, analysis of data, preparation of manuscript; YoungJun Ju, Akbar Ahmad, Ashley A. Untereiner, Zaid Altaany, Lingyun Wu: conduction of experiments; Csaba Szabo, Rui Wang: experimental design, interpretation of the data, preparation of manuscript.

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suggesting a physiological role for CSE-derived endogenous H_2S production in the *S*-sulfhydration of ATP synthase. Various forms of critical illness (including burn injury) upregulate H_2S-producing enzymes and stimulate H_2S biosynthesis. In liver tissues collected from mice subjected to burn injury, we detected an increased *S*-sulfhydration of ATP5A1 at the early time points post-burn. At later time points (when systemic H_2S levels decrease) *S*-sulfhydration of ATP5A1 decreased as well. In conclusion, H_2S induces *S*-sulfhydration of ATP5A1 at C244 and C294. This post-translational modification may be a physiological mechanism to maintain ATP synthase in a physiologically activated state, thereby supporting mitochondrial bioenergetics. The sulfhydration of ATP synthase may be a dynamic process, which may be regulated by endogenous H_2S levels under various pathophysiological conditions.

Graphical abstract



Keywords

H_2S; bioenergetics; *S*-sulfhydration; burn; ATP synthase; cysteine; mitochondria; hydrogen sulfide; burn injury

1. Introduction

The biology and pathobiology of the gasotransmitter hydrogen sulfide (H_2S) have received significant attention over the last decades. H_2S exerts broad range of physiological effects as an antioxidant, cytoprotective agent [1,2], vasorelaxant [3–5], neurotransmitter [6,7], and endogenous promoter of angiogenesis [8,9]. H_2S also plays important roles in the regulation of various inflammatory processes via a variety of mechanisms including the regulation of pro-inflammatory signaling [10–13]. Furthermore, H_2S plays a role in the control of metabolism, via the regulation of glucose and lipid metabolism, insulin sensitivity and energy balance [14–16].

During the last ten years the role of H_2S in the regulation of cellular bioenergetics has been completely re-evaluated. It has been known for several decades high concentrations of H_2S reversibly inhibit cytochrome c oxidase (complex IV) of the mitochondrial electron transport chain, resulting in the inhibition of ATP production and metabolic suppression [17].

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However, an additional body of more recent data, which has emerged over the last decade, demonstrates that H_2S, at lower concentrations, exerts a directionally different effect on mitochondria: it acts as a stimulator of mitochondrial bioenergetics and a mitochondrial protectant through a combination of several different mechanisms [18–35]. First, H_2S donates electrons to the mitochondrial electron transport chain through sulfide:quinone oxidoreductase (SQR) and mitochondrial Complex II, thereby stimulating oxidative phosphorylation (OXPHOS) and increasing mitochondrial ATP production [18–27]. Second, H_2S as a free radical scavenger can neutralize mitochondrial reactive oxygen and reactive nitrogen species, thereby maintaining mitochondrial integrity [28]. Third, H_2S inhibits the intramitochondrial phosphodiesterase 2A isoform, which, in turn, elevates intramitochondrial cAMP level; thus process stimulates mitochondrial electron transport, most likely via cAMP-dependent protein kinases, which phosphorylate key mitochondrial electron transport subunits [31]. Fourth, H_2S can stimulate mitochondrial DNA repair [32]. Fifth, H_2S can be metabolized to sulfite, sulfate and thiosulfate in the mitochondria; these species can act as endogenous storage 'pools' of H_2S (physiological "prodrugs") from which H_2S can be liberated [33–35].

ATP synthase (EC 3.6.3.14), also called mitochondrial Complex V, is localized in the mitochondrial inner membrane, and it is responsible for generating adenosine triphosphate (ATP). In order to produce ATP from adenosine diphosphate (ADP) and inorganic phosphate (Pi), ATP synthase requires energy. Carbon-based substrates, such as pyruvate and succinate provide $NADH/H^+$ and $FADH_2$ via the Krebs cycle in the mitochondrial matrix. These molecules donate electrons to mitochondrial Complex I and Complex II, fueling the mitochondrial electron transport chain; electrons flow through Complex III and finally complex IV, where they react with O_2 to form water. According to the theory of chemiosmosis, the purpose of this process is the pumping of protons from the mitochondrial matrix into the intermembrane space. This proton gradient is, in turn, "harvested" by ATP synthase via a rotatory mechanism to produce ATP [36–38].

Recent studies revealed a novel H_2S-induced posttranslational modification, termed protein *S*-sulfhydration (also known as S-persulfidation) [39–41]. During this process, the –SH group of cysteine residues become covalently converted to a –SSH group, which can result in changes in the activity of the protein. This process importantly contributes to physiological and pathophysiological H_2S- signaling. In 2010, Snyder's group, using liquid chromatography followed by tandem mass spectrometry (LC-MS/MS), identified 39 *S*-sulfhydrated proteins in liver lysates. These proteins include GAPDH, beta-tubulin, actin as well as mitochondrial ATP synthase [39]. We have decided to follow up on the last observation, in order to investigate the mechanisms and functional consequences of the sulfhydration of mitochondrial ATP synthase. The data presented in the current report confirm the sulfhydration of ATP synthase; identify the two regulatory cysteines involved and indicate that sulfhydration of ATP synthase serves as an activating post-translational modification and a stimulatory process of cellular bioenergetics.

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2. Materials and Methods

2.1. Materials

Sodium hydrosulfide hydrate ($NaHS \times H_2O$), neocuproine, deferoxamine mesylate salt (DFO), S-Methyl methanethiosulfonate (MMTS), polyvinylpyrrolidone were obtained from Sigma-Aldrich (St. Louis, MO). StartingBlock™ T20 (TBS) blocking buffer, Lipofectamine 2000, EZ-Link™ HPDP-biotin and Pierce™ streptavidin agarose were purchased from Thermo Fisher Scientific Inc. (Waltham, MA).

2.2. Cell culture

The human hepatocellular carcinoma-derived cell line (HepG2) and human embryonic kidney cells (HEK293) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cell cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 g/l glucose and 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 4 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin.

2.3. Animal handling

$CSE^{-/-}$ mice were generated as previously described [42]. For the mouse model of burn injury, male mice (10–12 weeks old) were used. All animal experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and approved by the local Animal Care Committee. Animals were maintained on standard rodent chow and had free access to food and water.

2.4. Biotin switch assay of *S*-sulfhydration

The biotin switch assay was carried out as described previously with some modifications [16]. Cells or liver tissue was homogenized in HEN buffer (250 mM Hepes-NaOH pH 7.7 supplemented with 1 mM EDTA, 0.1 mM neocuproine) containing 150 μM deferoxamine, 1% Nonidet-P40 (NP-40), and protease/phosphatase inhibitors. Homogenized samples were sonicated and centrifuged at 16,000 g for 15 min at 4°C. The supernatant samples were placed into 10K molecular weight concentrator and the retentates were used for protein determination. Lysates were diluted to reach 5.5 mg/ml final protein concentration and were added to HEN buffer supplemented with 2.5% SDS and 20 mM methyl methanethiosulfonate (MMTS). The samples were frequently shaked at 50°C for 25 min. Then, the MMTS was removed by adding acetone and in the meanwhile, the proteins were also precipitated at −20°C for 45 min. Pellets were resuspended in HEN buffer containing 1 % SDS and 4 mM biotin-N-[6-(biotinamido) hexyl]-3′-(2′-pyridyldithio) propinamide (HPDP). After 3-hour-long incubation at 25°C, biotinylated proteins were purified by streptavidin-agarose beads. The biotinylated proteins were eluted in 2× Laemmeli sample buffer and subjected to Western blotting with anti-ATP5A1 antibody (Abcam, Cambridge, MA, USA), anti-GAPDH antibody (Proteintech Group, Inc., Rosemont, IL) or anti-tetra His antibody (Qiagen, Hilden, Germany).

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2.5. Western blotting

Mouse liver tissue or cells were lysed (25 μg protein/10 μl) and diluted in Biorad 2× Laemmeli Sample Buffer. Samples were resolved on 4–12% NuPage Bis-Tris acrylamide gels (Thermo Fisher Scientific, Waltham, MA) and transferred to PVDF membranes. Membranes were blocked with 1% polyvinylpyrrolidone and then probed overnight with primary antibodies; such as (1) anti-ATP synthase 5A1 antibody (anti-ATP5A1, 1:1000, Abcam, Cambridge, MA), (2) anti-GAPDH antibody (anti-GAPDH, 1:1000, Proteintech Group, Inc., Rosemont, IL) or (3) anti-tetraHIS antibody (anti-His, 1:500, Qiagen, Hilden, Germany). On the following day, anti-goat (HRP, 1:3000, Abcam, Cambridge, MA) or anti-mouse-horseradish peroxidase conjugate secondary antibody (Sigma, St. Louis, MO) was applied. An enhanced chemiluminescent substrate (ECL, Pierce) was used to detect the signal using a CCD-camera-based chemiluminescence detection system or high sensitivity films (Amersham Hyperfilm ECL). The intensity of Western blot signals was quantified by densitometry using the ImageJ 1.45s software (NIH). The *S*-sulfhydrated samples were compared to the aliquots of lysate ("loading controls") that had not been subjected to the *S*-sulfhydration assay.

2.6. Site-directed mutagenesis

Human ATP5A1 cDNA construct was purchased from Sino Biological Inc. (Beijing, China). ATP5A1 cDNA was cloned into pGEM-T Vector/pCMV2-His tag. A single mutation at cyteine-244 or cysteine-294 in ATP5A1 was conducted using the Quick Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The oligonucleotides using for mutagenesis were 5′-GAAAAGAAGAAGCTGTACT**cc**ATTTATGTTGCTATTGGTC-3′ (forward) and 5′-GACCAATAGCAACATAAAT**gg**AGTACAGCTTCTTCTTTTC-3′ (reverse) for cysteine-244, and 5′-CCTTACTCTGGCT**c**TTCCATGGGAGAG-3′ (forward) and 5′-CTCTCCCATGGAA**g**AGCCAGAGTAAGG-3′ (reverse) for cysteine-294. The site-directed mutants were confirmed by DNA sequencing at the MOBIX Laboratory of McMaster University, ON, Canada.

2.7. ATP synthase activity measurement

A commercially available ATP synthase enzyme ELISA assay (Abcam, Cambridge, MA) was used with modifications to determined ATP synthase activity from cell lysates or from purified recombinant proteins. The kit is designed to immunocapture ATP synthase proteins using the same anti-ATP5A1 antibody that we previously applied for the biotin-switch *S*-sulfhydration assay. The immunocaptured ATP synthase enzyme operates in the "reverse" direction as the physiological role of ATP synthase: it hydrolyzes ATP to ADP and phosphate. This production of ADP is, in turn, coupled to the oxidation of NADH to NAD^+, which is monitored as a decrease in absorbance at 340 nm. ATP hydrolysis is inhibited by oligomycin (a specific inhibitor of ATP synthase that inhibits through binding to the F_o domain of the protein). During the cell lysate preparation, the entire ATP synthase enzyme is immunocaptured; we considered the oligomycin-inhibitable component of the ATP consumption as specific ATP synthase activity.

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2.8. Mouse model of burn injury

Male C57/BL6 mice (10–12 weeks old) were housed at 24–26°C on a 12:12 light: dark cycle. Burn injury was induced as described [43]. Sham mice and mice subjected to burn injury underwent identical experimental procedures except of injury. Following an intraperitoneal (i.p.) injection of 0.1 mg/kg buprenorphine, mice were anesthetized by inhalation of 3–5% isoflurane. Next, ~40% of the dorsum was shaved with electrical clippers and ~ 1 cc of lactated ringers (LR) solution was injected under the skin along the spinal column. The dorsa of burn treated animals were then exposed to ~95°C water for 10 sec to produce a full thickness scald wound covering ~30% of the total body surface area (TBSA). Mice were then resuscitated with 2 cc of LR. Burn and sham-treated mice were individually housed throughout the experimental period. To minimize animal suffering, pain or distress, animals were scored twice daily throughout the post-burn period using an IACUC-approved Rodent Intervention Score Sheet to assess their well-being and clinical status by a certified veterinarian. To minimize the suffering of the animals the analgesic buprenorphine was administered when indicated to reduce pain and distress. Cohorts of mice were sacrificed at 1, 4, 10, 20, and 30 days post-injury. Liver tissues were used for further analysis.

2.9. Statistical analysis

Data are shown as means ± SEM. Student's t-tests and one-way ANOVA with Dunnett post-hoc test were used to detect differences between groups; *$p < 0.05$ and **$p < 0.01$ considered statistically significant. All statistical calculations were performed using the Graphpad Prism 5 analysis software.

3. Results

3.1. H_2S induces the *S*-sulfhydration of the ATP synthase alpha subunit

The *S*-sulfhydration of ATP synthase was confirmed both in HepG2 and HEK293 cell lysates utilizing a biotin switch *S*-sulfhydration assay. The α subunit of the F1 domain (ATP5A1), which – together with the ß subunit – is indispensable for the catalytic activity of the enzyme [36–38] – was *S*-sulfhydrated. ATP5A1 sulfhydration increased in response to treatment of the cell lysates with NaHS (50 – 300 μM) (Fig. 1).

3.2. H_2S increases ATP5A1 activity

Next, we measured the specific activity of ATP synthase with and without NaHS treatment. ATP synthase isolated from both HEK and HepG2 cell lysates showed a concentration-dependent increase of its enzymatic activity in response to NaHS (10 – 100 nM). The effect of NaHS – similar to many other pharmacological effects of H_2S – followed a bell-shaped concentration-response curve: at higher concentrations (1–10 μM) the activation was no longer apparent, and a tendency for an inhibition of enzymatic activity was noted (Fig. 2).

3.3. H_2S induced *S*-sulfhydration of ATP5A1 occurs at cysteines 244 and 294

There are two highly conserved cysteine (Cys) residues, Cys 244 or Cys 294, in ATP5A1 (Fig. 3). To determine the cysteines of ATP5A1 that are subject to *S*-sulfhydration, we mutated Cys 244 and Cys 294 to serine via site-directed mutagenesis. HEK293 cells were

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then transfected with plasmids encoding three different recombinant mutants (C244S; C294S; C244S and C294S double-mutant). NaHS-induced *S*-sulfhydration was abolished in HEK293 cells expressing the recombinant double mutant ATP5A1 (C244S/C294S) (Fig. 4).

3.4. Double mutation of Cys 244 and 294 suppresses ATP synthase enzyme activity

Comparison of the activity of the HIS-tagged labeled recombinant proteins (C244S; C294S; C244S/C294S) from HEK293 cell lysates demonstrated that double mutant recombinant protein (C244S/C294S) exhibits significantly reduced enzyme activity (over 50% suppression of activity), compared to the single cysteine mutated versions, which exhibit a slight reduction in activity (Fig. 5).

3.5. Basal ATP5A1 *S*-sulfhydration is reduced CSE-KO mice

Wild-type mice exhibited a detectable basal level of S-sulfhydration of ATP5A1; ATP5A1 sulfhydration was significantly lower in liver homogenates harvested from $CSE^{-/-}$ mice than ATP5A1 sulfhydration in wild-type mice (Fig. 6).

3.6. *S*-sulfhydration of ATP5A1 is increased after burn injury

Recent studies demonstrate that CSE is upregulated and H_2S plasma levels are increased in mice subjected to burn injury [44,45]. In liver homogenates obtained from mice subjected to burn injury, increased *S*-sulfhydration of ATP5A1 was detected at the early time points, followed by a return to baseline over time (Fig. 7). *S*-sulfhydration of GAPDH [39] – a positive control in our *S*-sulfhydration assay – exhibited a similar time-course (Fig. 7).

4. Discussion

The main conclusions of the current study are the following: **(a)** confirming and extending on a the prior observation [39] that ATP synthase is a subject of sulfhydration by H_2S, we have demonstrated that the alpha subunit of ATP synthase (ATP5A1) becomes sulfhydrated in response to the H_2S donor NaHS; **(b)** ATP5A1 sulfhydration occurs at Cys 244 and Cys294; **(c)** H_2S, at low concentrations, stimulates the activity of ATP synthase; **(d)** mutation of either cysteine 244 or 294 slightly decreases the catalytic activity of ATP synthase, while mutation of both cysteines results in a marked inhibition; **(e)** *in vivo*, the alpha subunit of ATP synthase is basally sulfhydrated; **(f)** the basal sulfhydration of ATP synthase is, at least in part, due to CSE-derived H_2S, as it is suppressed in $CSE^{-/-}$ mice; and, finally, **(g)** burn injury, which upregulates CSE and increases H_2S production, results in an increase in ATP synthase sulfhydration.

Recent work has identified several distinct H_2S-related signaling mechanisms. First, H_2S can react with metal centers by binding and transferring electrons. For example, cytochrome c oxidase (Complex IV) contains two heme molecules, a cytochrome ‘a’ and cytochrome ‘a3’, and two copper centers, the CuA, and CuB centers. Reaction of these centers with H_2S results in the inhibition of the activity of Complex IV, leading in mitochondrial “poisoning”. This mechanism is generally accepted as the principal mechanism of toxicity of high concentrations of H_2S [18,19]. Second, H_2S can neutralize reactive oxygen and nitrogen species via direct as well as indirect mechanisms. H_2S can directly act as a free radical and

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oxidant scavenger, although with relatively slow reaction rates; and it can also upregulate endogenous cellular antioxidant responses, for instance via the regulation of the NRF2 response [2,28,40]. Via these mechanisms, H_2S can exert cytoprotective and mitochondrial protective effects. A third pathway of H_2S signaling occurs via post-transcriptional modulation of reactive cysteine residues via protein *S*-sulfhydration (also termed S-persulfidation) [39–41,46]. During the *S*-sulfhydration, H_2S covalently modifies the SH groups of cysteines, creating persulfides (-SSH groups). Cysteines can be subject to several different oxidative posttranslational modifications (oxPTMs) (similar to S-nitrosylation, S-sulfonation, S-sulfenylation, and S-glutathionylation) in addition to S-sulfhydration. Persulfide formation does not occur through direct interaction between H_2S and the –SH group of cysteines, because sulfur is in its lowest oxidation state, −2, in both H_2S and –SH group. Therefore, polysulfides have been identified as the chemical species that are directly responsible for protein sulfhydration [41,46]. Nevertheless, in *in vitro* experimental systems, *S*-sulfhydration reactions can be produced by chemical H_2S donors [39–41,46–52], presumably because these donors lead to the formation of polysulfide species, which, in turn, result in the formation of the *S*-sulfhydrated cysteine groups on various protein targets. Several proteins have been identified as targets of *S*-sulfhydration by H_2S. The effect of S-sulfhydration on protein function depends on the pH and the position of the target cysteine amino acids in the protein (e.g. its proximity to the active centrum of the enzyme), the surrounding amino acids, local electric charge and redox status [41,46]. The current report confirms that the ATP5A1 is a *S*-sulfhydration target and indicate that the functional consequence of this reaction is the stimulation of ATP synthase activity. It remains to be further elucidated whether the S-sulfhydration of ATP synthase is transient or permanent; to our knowledge the reversibility of S-sulfhydration reactions under cellular or physiological conditions has not yet been explored in the literature.

Several groups, including our own, have previously characterized the effect of H_2S on mitochondrial activity and cellular bioenergetics [18,19,22–27]. These studies demonstrated that H_2S induces a bell-shaped bioenergetic concentration-response in various cell types *in vitro*, with activation at lower concentrations and inhibition at higher concentrations. In followup studies different parts of the mitochondrial electron transport chain were separately tested. When Complexes I–IV or Complexes II-IV were functional, the bell-shaped effect of H_2S persisted; when Complex IV alone was functional, the activating effect of H_2S was not seen at lower concentrations, but the inhibitory effect at higher concentrations persisted [27]. In these experiments, however, ATP synthase activity was not measured; the assay used (Extracellular Flux Analysis) measures oxygen consumption, and not ATP production. In other experiments, however, we have also measured ATP generation [24] and showed that NaHS increases it. It would be important to measure the effect of H_2S on ATP synthase activity directly in a cellular or mitochondrial preparation; however, unfortunately such assays are currently beyond our technical capabilities. The assay shown in the current paper (which is based on ATP synthase activity measurement using an ELISA system), nevertheless, indicate that H_2S has a direct effect on the catalytic activity of ATP synthase.

We have noted in the current study that that there is a significant difference in the concentration-responses to H_2S when using whole cell-homogenate-based assays vs. isolated/recombinant enzymes or similar reductionist systems. Based on prior studies in the

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literature, we believe that the difference between the cell-homogenate-based potency of H_2S and the enzyme-based potency of H_2S is due to the chemical and pharmacological nature of H_2S. When cell-homogenates or whole-cell-based systems are used, first of all, the indicated H_2S concentrations only represent nominal and initial concentrations. For instance, in cell-based studies, H_2S concentrations will diminish by the time H_2S reaches the cells at the bottom of the culture dish, and they will further diminish as they reach the cytosolic – and especially the mitochondrial – compartment. This is believed to happen due to passive degradation/oxidation as well as active metabolism of H_2S though various metabolic pathways (e.g. rhodanese). Similarly, in whole cell homogenates, the H_2S applied to the homogenate is subject to degradation and reactions by multiple enzymes and non-enzymatic processes, which diminishes its actual concentration that is "seen" by the target enzyme of the experiment. So far, to our knowledge, no investigators have been able to reliably quantify, in absolute terms, the intracellular (e.g. mitochondrial) H_2S levels that one can reach when applying exogenous H_2S donors (or even when applying mitochondrially targeted ones). We can, nevertheless, assume that the concentrations that ATP synthase "sees" in a whole cell homogenate are markedly lower than the initial (applied) concentrations of H_2S. In contrast, in systems where H_2S donors are directly mixed with recombinant or isolated enzymes dissolved in simple solutions, the actual H_2S concentrations that the enzyme "sees" are likely to be much closer to the initially applied (nominal) concentrations. In the current paper we find that the concentrations of H_2S needed to *S*-sulfhydrate ATP synthase, when cell homogenates were treated with H_2S, was approximately 100 μM, while the concentrations of H_2S needed to activate immunocaptured H_2S in a reductionist *in vitro* system was approximately 0.1 μM (a 1000-fold concentration difference). Similarly, we have previously demonstrated that the concentration of H_2S needed to inhibit Complex IV activity in whole mitochondrial preparation was 100 μM, while the concentration of H_2S needed to inhibit purified mitochondrial Complex IV were 0.1 μM [27] (again, a 1000-fold concentration difference). Taken together, the above considerations, together with the finding that mutation of the *S*-sulfhydrated cysteines diminishes the catalytic activity of ATP synthase are consistent with the suggestion that there is a causative link between the sulfhydration of ATP synthase and its catalytic activation by H_2S.

Previous studies have demonstrated that low concentrations of H_2S can stimulate mitochondrial function via a variety of mechanisms (Fig. 8). H_2S (a diffusible molecule) can enter the mitochondrial compartment either via diffusion after it is produced by cytosolic enzymes – CSE, or cystathionine beta synthase (CBS), or 3-mercaptopyruvate sulfurtransferase (3-MST) – or by constitutive mitochondrial H_2S-producing enzymes (3-MST and CBS). Moreover, CSE and CBS can also translocate into the mitochondria under various pathophysiological conditions [20,22]. Once in the mitochondrial compartment, H_2S can stimulate mitochondrial function via a number of mechanisms outlined in the Introduction section [18–35]. Based on the results of the current study, H_2S, via *S*-sulfhydration of ATP synthase (Complex V), can also contribute to the activation of mitochondrial function. The latter effect may be necessary to optimize mitochondrial efficiency, because if H_2S, through electron donation [18–25] and/or through the stimulation of intramitochondrial cAMP levels [31], increases electron transport, this is expected to

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produce an increase in the proton gradient across the mitochondrial inner membrane. It makes, therefore, biological sense that an increase in the specific activity of ATP synthase on the "receiving end", balances these processes, in order to efficiently "harvest" the increased proton gradient. All of these processes are shown in Fig. 8. For reasons of completeness, Fig. 8 also indicates the inhibitory effect of H_2S on cytochrome c oxidase (complex IV) [17,27]; this process occurs at higher (pathophysiological or toxicological) levels of H_2S.

Our data showing that S-sulfhydration of ATP synthase is lower in the $CSE^{-/-}$ mice presented indicate that basal S-sulfhydration of ATP synthase may be a physiological mechanism in response to endogenously produced H_2S. In addition to CSE-derived H_2S, mitochondrial H_2S levels are also affected by 3-MST (a partially mitochondrially localized enzyme), as well as CBS (which is physiologically present in liver mitochondria) [22,23,27]. It is conceivable, therefore, that the production of H_2S by 3-MST and/or CBS may also contribute to the sulfhydration of ATP synthase under physiological (or pathophysiological) conditions; this remains to be tested in future studies. Mitochondrially targeted H_2S donors are also available now for *in vitro* and *in vivo* studies, for instance the compound AP39; this compound is known to increase cellular bioenergetics *in vitro* [30]. Further work needs to be conducted to test whether mitochondrial delivery of H_2S increases the sulfhydration and catalytic activity of ATP synthase.

Previous work has already examined the functional consequences of various modifications of the cysteines in ATP synthase. Cys 294, which is located on the surface of ATP synthase protein, has been shown to form an acid–base motif and is easily targeted by various external redox stimuli. Moreover, Cys 244 and Cys 294 have been shown to form a disulfide bond under oxidative stress conditions [54–56]. In addition – similar to the results of the current study – Wang and colleagues have previously demonstrated that the catalytic activity of the α subunit of ATP synthase is decreased either after the mutation of Cys 244 or in response to the mutation of Cys 294 [54]. Cys 244 is now being viewed as a general 'redox sensor' of ATP synthase, which plays a key role in the regulation of the activity of ATP synthase under various physiological and pathophysiological conditions [55]. The exact (patho)physiological consequence(s) of the sulfhydration of ATP synthase – in view of the fact that the same cysteine can also be a target for a variety of other post-translational modifications – remains to be further explored in future studies.

We are aware of the fact that the current study has a number of limitations. First, the exact mechanism of the sulfhydration process (via H_2S itself, or, more likely, via intermediary persulfide reactions) remains to be characterized. Second, a difference was noted between the concentrations of H_2S needed to *S*-sulfhydrate ATP synthase and the concentrations needed to activate the enzyme. Although this concentration difference may be explained by the difference in the experimental conditions used, further work may be necessary to further address this issue. Third, we have not investigated the reversibility of the S-sulfhydration reaction. Fourth, we have not distinguished between the activating effect of H_2S on ATP synthase activity (occurring at lower concentrations) and the inhibitory effects seen at higher concentrations. Fifth, the mechanism(s) and source(s) of H_2S responsible for the endogenous sulfhydration of the enzyme remain to be identified; the experiments using $CSE^{-/-}$ mice indicate that CSE plays an important role; the roles of the other two H_2S-generating

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enzymes (CBS and 3-MST) remain to be explored. Sixth, the role of the increased sulfhydration of ATP synthase – in light of the complex pathomechanisms of burn injury, and the multiple potential roles of H_2S in burns [57] – remain to be characterized. Seventh, the current *ex vivo* studies only investigated one selected organ (the liver); it remains to be examined whether *S*-sulfhydration of ATP synthase occurs in other organs as well, and if it does, what functional role does it play. Eight, we have only studied one, selected pathophysiological condition (burn injury). Given the important changes in H_2S homeostasis in various disease states with either increased or decreased H_2S production, the potential changes in the sulfhydration of ATP synthase in various pathophysiological conditions remain to be explored in future studies.

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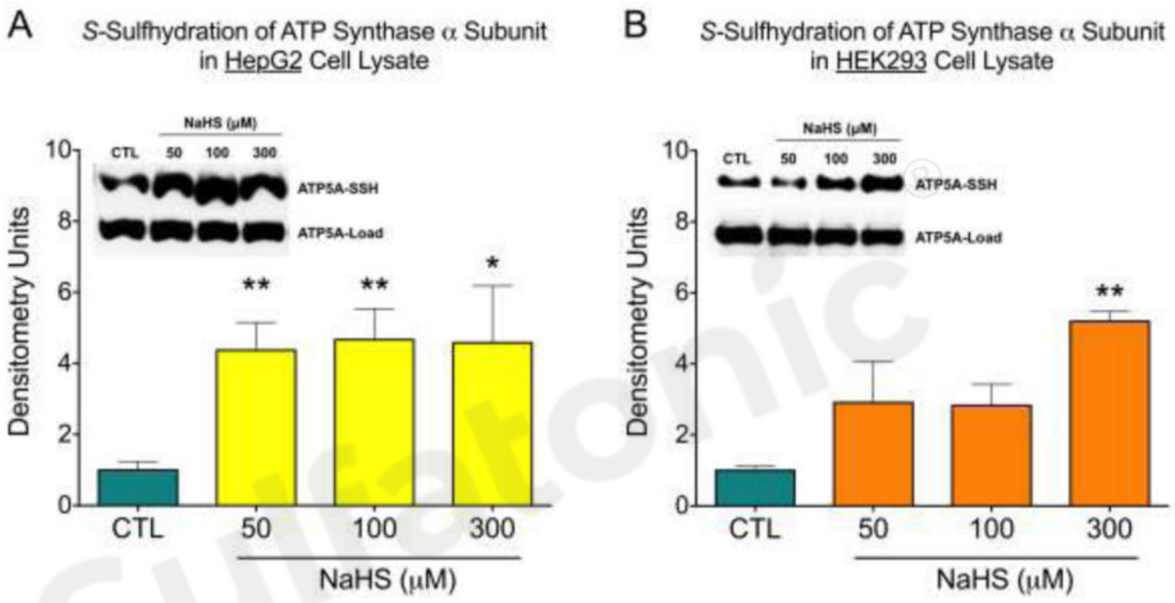


Figure 1.

H_2S induced *S*-sulfhydration of ATP5A1. HepG2 (**A**) and HEK 293 (**B**) cell lysates were incubated with NaHS at various concentrations (50–300 μM) for 30 min at 37°C. *S*-sulfhydration of ATP5A1 was detected by the biotin switch assay using anti-ATP5A1 antibody. Densitometry data show mean±SEM of n=5 independent experiments, *p<0.05 and **p<0.01 indicate significantly increased *S*-sulfhydration after NaHS, compared to the vehicle control (CTL) group.

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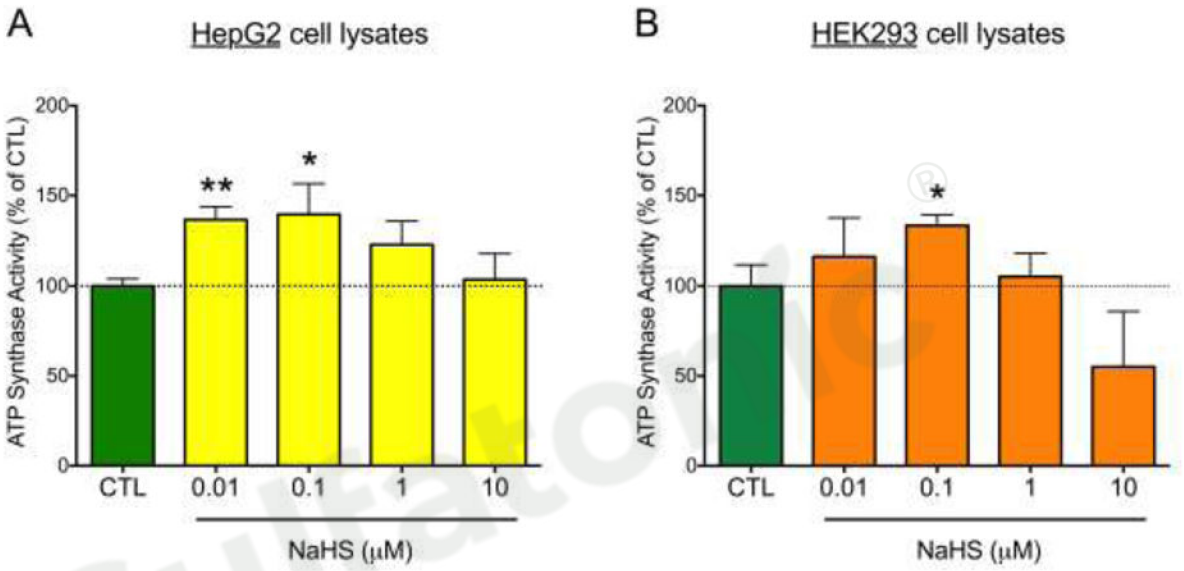


Figure 2.

NaHS stimulates ATP5A1 enzyme activity in HepG2 (**A)** and HEK293 (**B)** cell lysates. ATP synthase protein, immunocaptured from the cell homogenates, was incubated with NaHS for 30 min at 37°C, and enzyme activity was determined by a kinetic, spectrophotometric assay which measures the consumption of ATP and the consequent production of ADP (which is coupled to the oxidation of NADH to NAD+). The oligomycin-inhibitable component of the ATP consumption was considered specific ATP synthase activity. Data were expressed as percent values of control (CTL) the ATP synthase activity (immunocaptured ATP synthase treated with NaHS vehicle). Data show mean±SEM values from n=3 independent experiments. *p<0.05 and **p<0.01 indicate the stimulatory effect of NaHS on ATP synthase.

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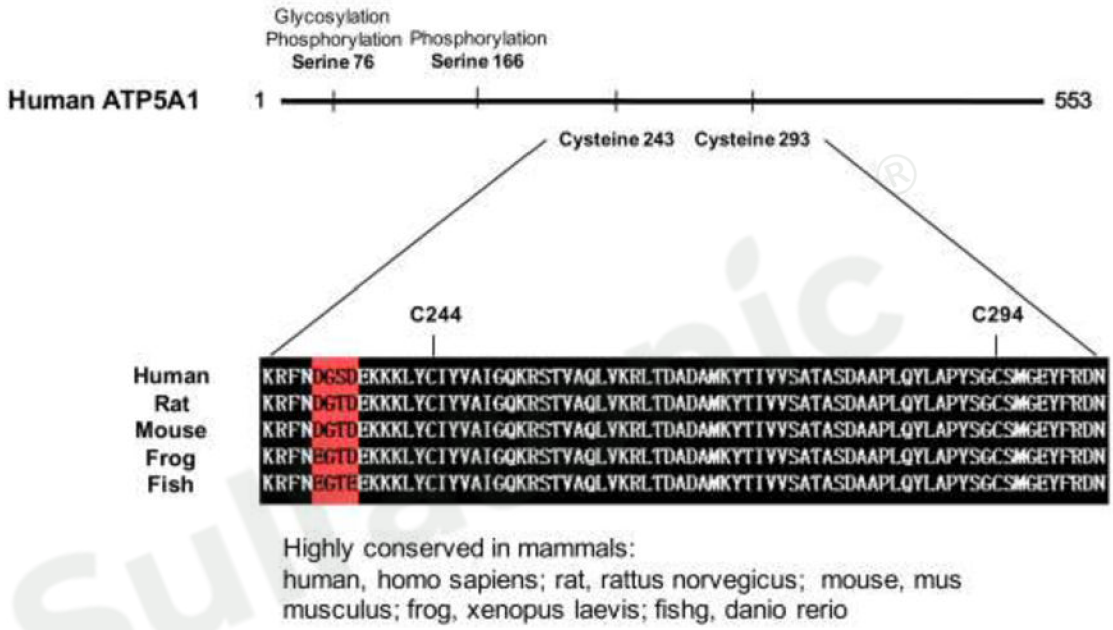


Figure 3.
Domains of ATP5A1, indicating the sites of highly conserved cysteine residues across different species.

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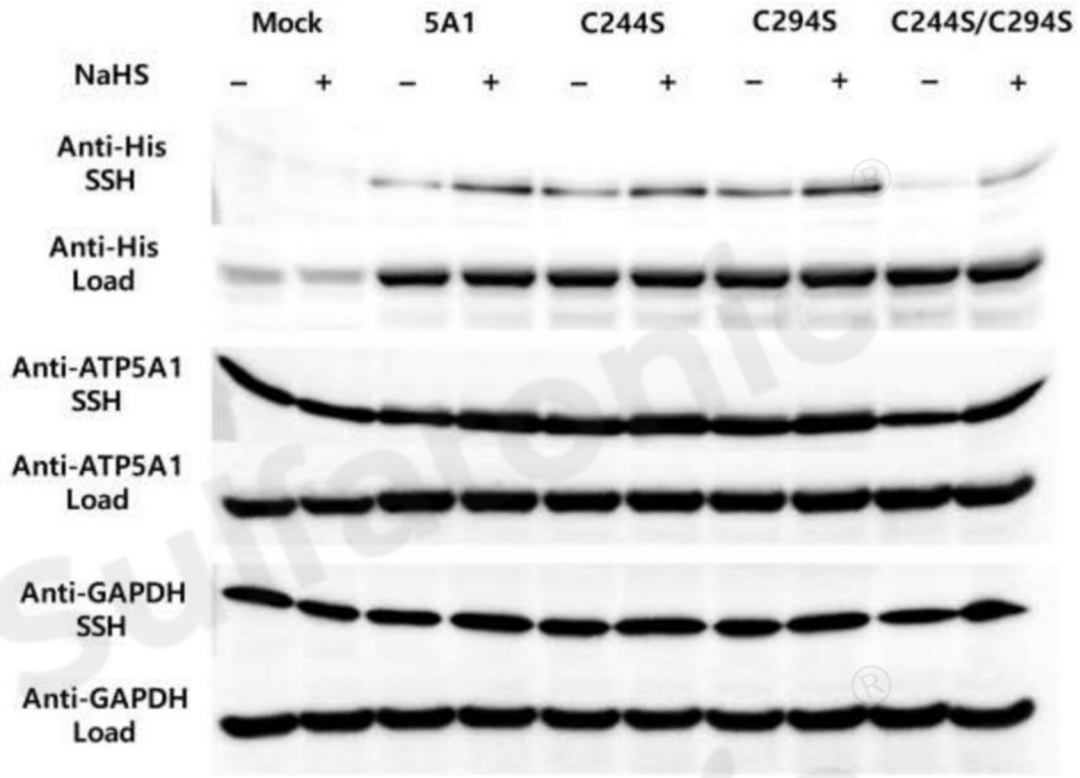


Figure 4.

Western blot analysis of HEK-293 cells transfected either with empty vector, or native human ATP5A1 (5A1), or cysteine 244 mutant (C244S) human ATP5A1, or cysteine 294 mutant (C294S) human ATP5A1, or the double mutant C244S/C294S human ATP5A1 plasmids for 48 hrs. Cell lysates were incubated with NaHS (100 μM) for an additional 30 min at 37°C. The biotin switch assay using anti-tetraHis antibody was used to differentiate the recombinant ATP5A1 from the endogenous ATP5A1.

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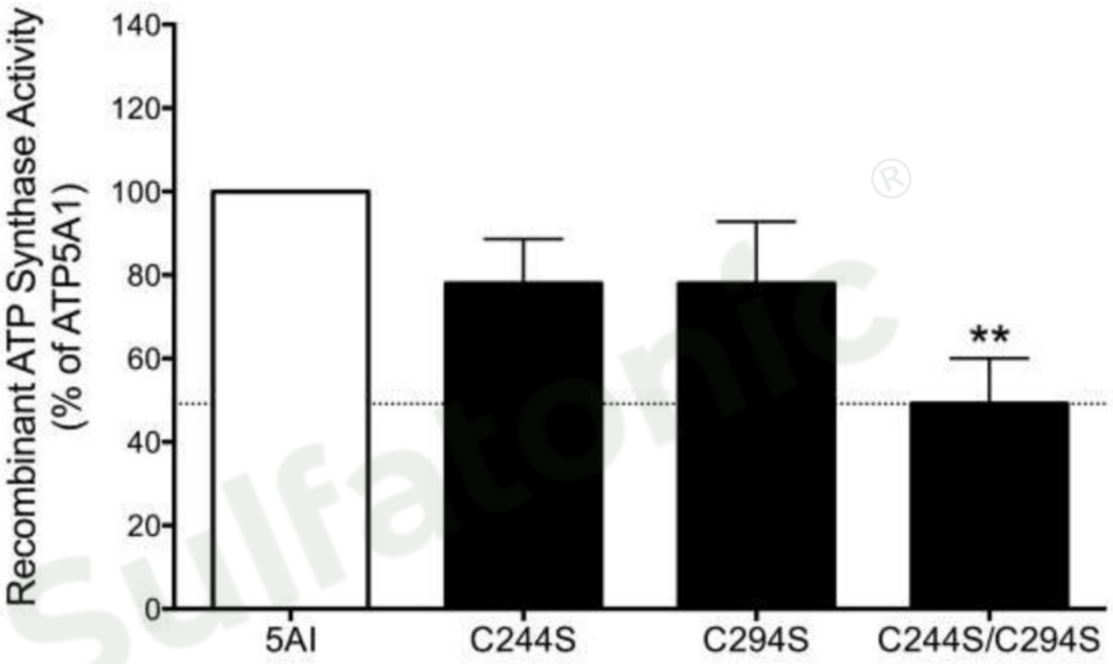


Figure 5.

The double mutant recombinant protein (C244S/C294S) exhibits a significantly reduced enzyme activity compared to the intact human ATP5A1 protein. Mean±SEM values are shown from n=3 independent experiments. **p<0.01 indicates significant suppression of the catalytic activity of the double mutant protein, compared to wild-type protein.

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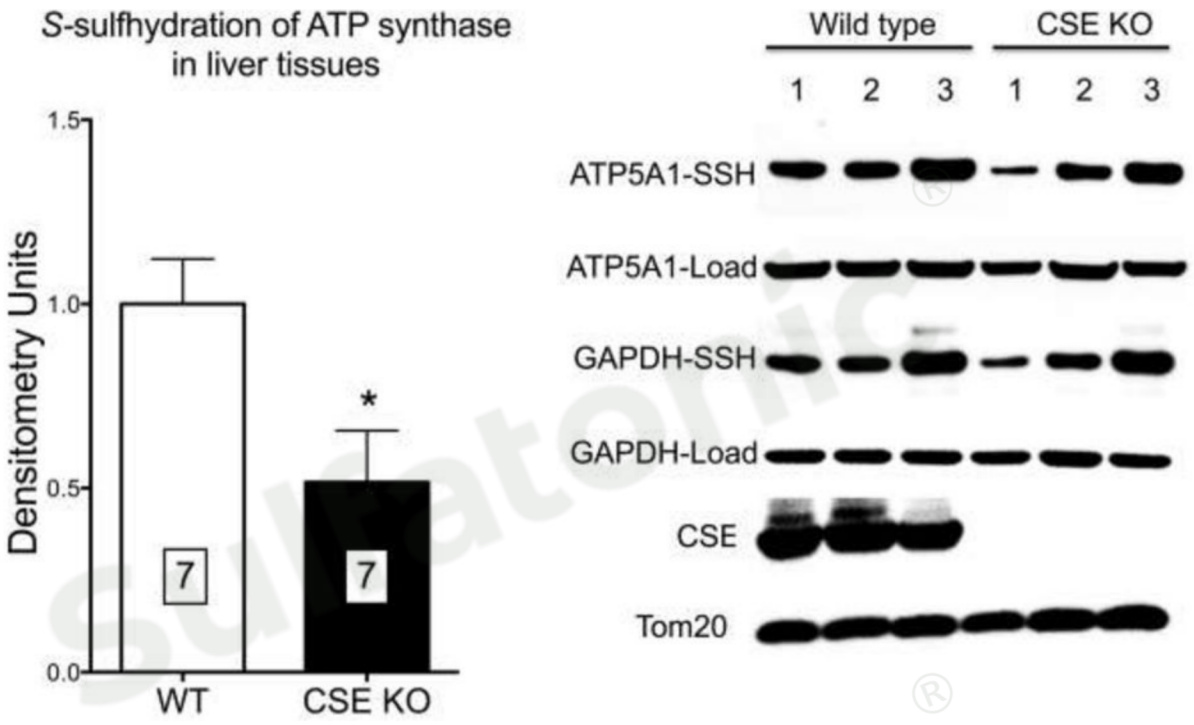


Figure 6.

ATP5A1 prepared from livers of CSE$^{-/-}$ mice exhibits reduced *S*-sulfhydration compared to ATP synthase prepared from livers of wild-type mice. Densitometry analysis represents mean±SEM values of n=7 wild-type or n=7 CSE$^{-/-}$ livers. *p<0.05 shows significant difference between the CSE$^{-/-}$ and the wild-type group.

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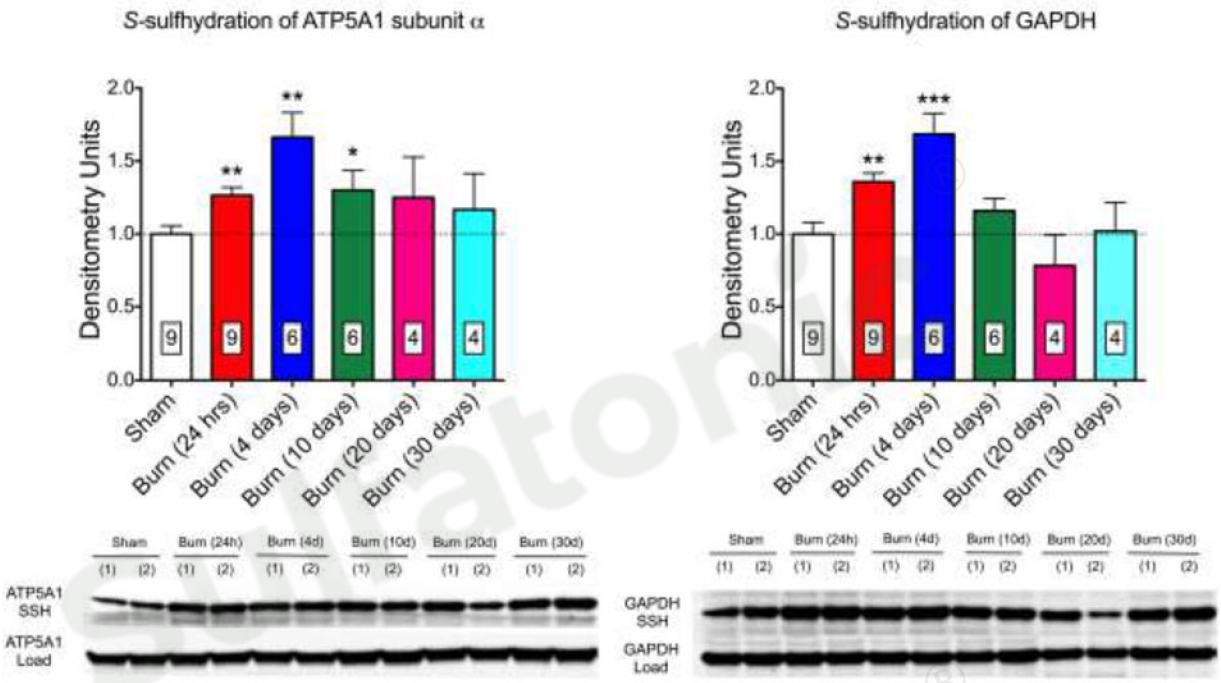


Figure 7.

S-sulfhydration of ATP5A1 **(A)** and GAPDH **(B)** is increased at early time points after burn injury in livers of mice. A representative western blot and densitometry analysis (mean ±SEM) of n=4–9 liver tissues is shown. *p<0.05 and **p<0.01 represent significant increases in the sulfhydration of the target enzyme after burn injury compared to the sham group.

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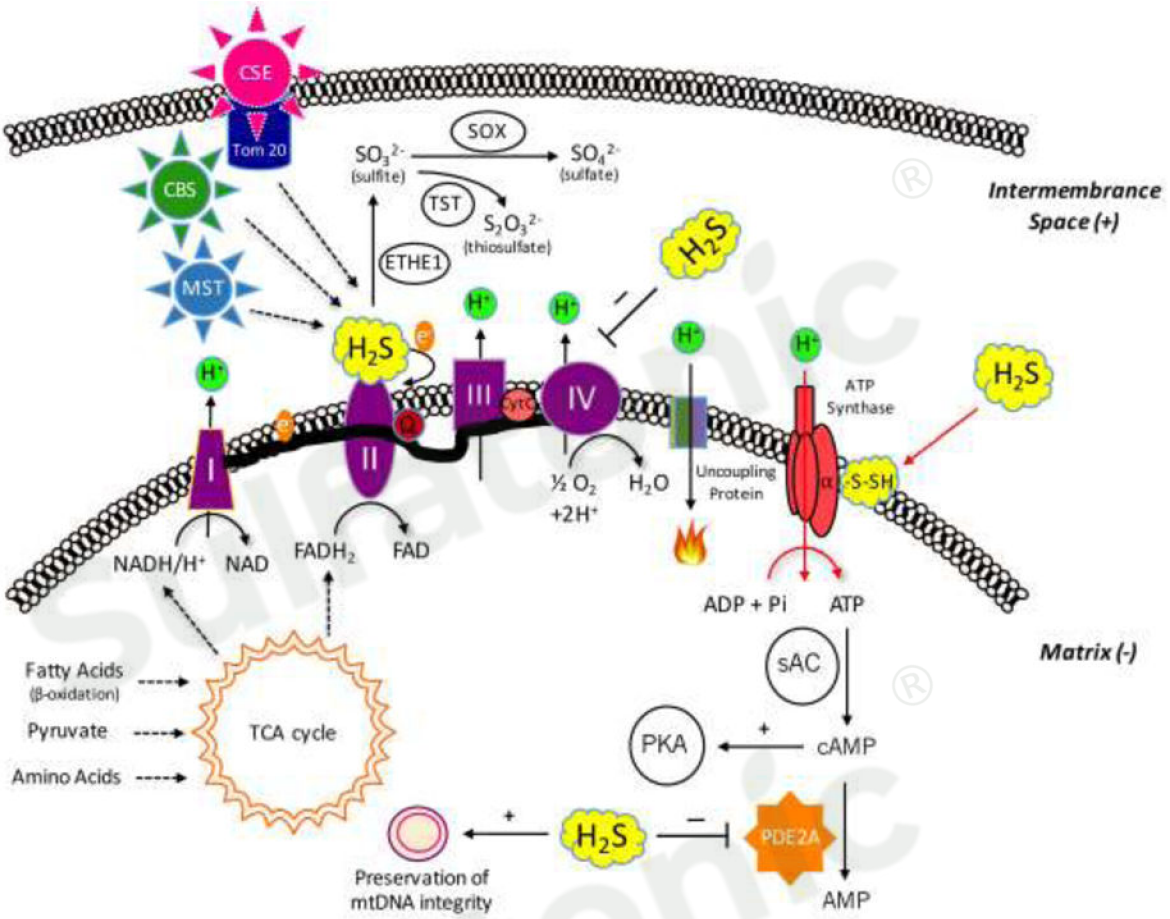


Figure 8.

Summary of the various effects of H_2S on mitochondrial bioenergetics. **(a)** The novel findings contained in the current report (*S*-sulfhydration of ATP synthase) are indicated with red arrows. H_2S can be produced in the mitochondrion constitutively by two distinct H_2S-producing enzymes, 3-MST, and CBS. Moreover, CSE is capable of translocating to the mitochondrial outer membrane under certain stress condition (e.g.: increased intracellular calcium signal), contributing the growth of intramitochondrial H_2S level. Lower concentrations of H_2S exert stimulatory effects on mitochondrial function via several different mechanisms: **(b)** H_2S can donate electrons to mitochondrial electron transport chain. **(c)** H_2S can act as an antioxidant neutralizing mitochondrial-derived reactive oxygen and nitrogen species, stabilizing the electron transport chain proteins, as well as preventing mitochondrial DNA damage. **(d)** H_2S oxidization can results in sulfite, sulfate and thiosulfate; some of these species can also act as "pools" or sources of biologically active H_2S. **(e)** H_2S can inhibit mitochondrial PDE2A enzyme, which increases intramitochondrial cAMP levels, and stimulates mitochondrial function via activation of the cAMP-dependent protein kinase (PKA). Higher concentrations of H_2S can also exert marked inhibitory effects on mitochondrial function: **(f)** H_2S can inhibits cytochrome c oxidase (complex IV) resulting in a reversible inhibition of mitochondrial electron transport and ATP production.

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