Molecular and Cellular Endocrinology xxx (2018) 1–9

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Endogenous H_2S resists mitochondria-mediated apoptosis in the adrenal glands via ATP5A1 S-sulfhydration in male mice

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ARTICLE INFO

Article history:
Received 14 November 2017
Received in revised form
21 February 2018
Accepted 22 February 2018
Available online xxx

Keywords:
Adrenal
Mitochondria
Apoptosis
Hydrogen sulfide
ATP5A1
S-sulfhydration

ABSTRACT

In a previous study, we showed that endogenous hydrogen sulfide (H_2S) plays a key role in the maintenance of intact adrenal cortex function via the protection of mitochondrial function during endoxemia. We further investigated whether mitochondria-mediated apoptosis is involved in H_2S protection of adrenal function. LPS treatment resulted in mitochondria-mediated apoptosis in the adrenal glands of male mice, and these effects were prevented by the H_2S donor GYY4137. In the model of Y1 cells, the LPS-induced mitochondria-mediated apoptosis and blunt response to ACTH were rescued by GYY4137. The H_2S-generating enzyme cystathionine-β-synthase (CBS) knockout heterozygous ($CBS^{+/-}$) mice showed mitochondria-mediated apoptosis in the adrenal gland and adrenal insufficiency. GYY4137 treatment restored adrenal function and eliminated mitochondria-mediated apoptosis. Maleimide assay combined with mass spectrometry analysis showed that a number of proteins in mitochondria were S-sulfhydrated in the adrenal gland. ATP5A1 was further confirmed as S-sulfhydrated using a modified biotin switch assay. The level of S-sulfhydrated ATP5A1 was decreased in the adrenal gland of endotoxemic and $CBS^{+/-}$ mice, which was restored by GYY4137. ATP5A1 was identified as sulfhydrated at cysteine 244 by H_2S. Overexpression of the cysteine 244 mutant ATP5A1 in Y1 cells resulted in a loss of LPS-induced mitochondria-mediated apoptosis and GYY4137 restoration of LPS-induced hyporesponsiveness to ACTH. Collectively, the present study revealed that decreased H_2S generation leads to mitochondrial-mediated apoptosis in the adrenal cortex and a blunt response to ACTH. S-sulfhydration of ATP5A1 at cysteine 244 is an important molecular mechanism by which H_2S maintains mitochondrial function and steroidogenesis in the adrenal glands.

1. Introduction

Reversible adrenal insufficiency has frequently been diagnosed in critically ill sepsis patients with either low basal cortisol levels or adrenocortical hyporesponsiveness to adrenocorticotropic hormone (ACTH) stimulation (Neary and Nieman, 2010; Tsai et al., 2006). To date, there is no effective therapy for reversible adrenal insufficiency (RAI) outside of glucocorticoid (GC) supplementation therapy. However, GC supplementation is also limited due to its adverse effects on insulin resistance, protein catabolism, and immunosuppression (Marik et al., 2008; Lipiner-Friedman et al., 2007). Thus, alternative therapeutic strategy for management of adrenal insufficiency during sepsis are urgently required.

Hydrogen sulfide (H_2S) has recently been suggested as "the third endogenous gaseous signaling transmitter" in mammalian tissues. H_2S has been implicated in many physiological and pathological processes, including vasodilation (Olson et al., 2010), inflammation (Mani et al., 2014), angiogenesis (Qipshidze et al., 2012) and cytoprotection (Calvert et al., 2010) in various tissues. H_2S synthesis from L-cysteine naturally occurs in a range of mammalian tissues mainly through the activity of the enzymes, including cystathionine-γ-lyase (CSE, EC 4.4.1.1) and cystathionine-β-synthetase (CBS, EC 4.2.1.22) (Wang, 2003). In previous studies, we demonstrated that both CSE and CBS are expressed in the adrenal cortex, and decreased H_2S resulting from the downregulation of CBS and

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https://doi.org/10.1016/j.mce.2018.02.011
0303-7207/

CSE contributes to adrenal insufficiency during endoxemia in mice (Wang et al., 2014), suggesting that H_2S replacement could be a potential therapeutic strategy for endotoxemia-associated adrenal insufficiency.

An increasing body of evidence indicates that increased apoptotic processes play a determining role in the outcome of sepsis syndromes (Hotchkiss et al., 1999, 2001; Jia et al., 2004). Mitochondrial-mediated apoptosis, a pathway of apoptotic death in mammals (Bouillet et al., 1999), occurs due to cytochrome c (Cyt-c) release from mitochondria to the cytoplasm. In the cytoplasm, Cyt-c stimulates the apoptosome formation, which proteolytically activates procaspase-3 to finally induce apoptosis (Schimmer et al., 2001; Kluck et al., 1997). Meanwhile, Cyt-c release is caused by increased mitochondrial outer membrane permeabilization, which results from the interaction of Bax with truncated Bid (t-Bid) in the mitochondrial outer membrane (Er et al., 2006). A number of studies have demonstrated that mitochondrial-mediated apoptosis is involved in the pathogenesis of sepsis-associated tissue injuries (Chang et al., 2007; Chen et al., 2017; Haendeler et al., 1996). However, whether adrenal insufficiency is associated with mitochondrial-mediated apoptosis in the adrenal gland during sepsis remains largely unknown.

We previously showed that mitochondrial damage contributes to adrenal insufficiency caused by endotoxemia and H_2S replacement restores adrenal function and repairs mitochondrial damage in the adrenal gland (Wang et al., 2014). A recent study reported that H_2S supplementation markedly inhibits mitochondria-mediated endothelial cell apoptosis caused by high salt insult (Zong et al., 2015). Thus, we investigated the association of mitochondria apoptosis with adrenal insufficiency and examined the recovery effect of H_2S on mitochondria apoptosis in the adrenal gland during endotoxemia. Given that H_2S predominantly signals by the S-sulfhydrating cysteines of its target proteins, we identified the key proteins modified by H_2S in mitochondria. Here, we showed that endogenous H_2S S-sulfhydrates ATP5A1 at cysteine 244 and subsequently enhances its activity to prevent mitochondria-mediated apoptosis, thereby maintaining the function of the adrenal cortex.

2. Materials and methods

2.1. Animals and experimental protocols

Adult male C57BL/6 mice, weighing 17–19 g, were purchased from the Shanghai SLAC Laboratory Animal Co. (Shanghai, China). $CBS^{+/-}$ mice on a C57BL/6J background were originally obtained from the Jackson Laboratory (Bar Harbor, ME). Male $CBS^{+/-}$ mice were used in the present study. All animals were housed with regular light-dark cycles (lights on at 7:00 AM, lights off at 7:00 PM.) under a controlled temperature (22 ± 2 °C) and humidity ($50 \pm 10\%$) and were provided standard diet and water ad libitum. All animal protocols were approved by the ethics committee of Experimental Animals of Second Military Medical University.

Adult male C57BL/6 mice were randomly divided into the following groups: control, LPS and LPS + GYY4143. Purified LPS extracted from the membrane of *Escherichia coli* 0111:B4 (Sigma-Aldrich, St. Louis, Missouri, USA) was dissolved in sterile pyrogen-free saline and injected i.p. at a dose of 5 μg/g at 16:00–16:30 PM. GYY4137 (Cayman Chemical, Ann Arbor, MI), the slow-releasing H_2S donor, was dissolved in sterile saline and injected i.p. immediately before the injection of LPS at a dose of 50 μg/g. The control group received an equivalent volume of saline at 16:00–16:30 PM. Adult male $CBS^{+/-}$ mice were randomly divided into two groups: vehicle and GYY4143. GYY4143 was injected i.p. at a dose of 50 μg/g, whereas the mice in the control group received an equivalent volume of saline at 16:00–16:30 PM. Male wild-type mice from the same litter of $CBS^{+/-}$ mice served as control mice.

2.2. Cell culture

Y1 cells obtained from Chinese Academy of Sciences were maintained in DMEM medium containing 5% FBS at 37 °C in 5% CO_2-95% air. The cells were plated in twelve-well plate at a density of 4.5×105 cells/well and cultured in the above media at 37 °C in 5% CO_2-95% air. After incubating for 12 h, the cells were treated with LPS (100 ng/ml) with or without GYY4137 (50 μM) for 24 h. The cells were then fixed in 4% paraformaldehyde for TUNEL assay or harvested for western blotting.

2.3. ACTH stimulation test

Corticosterone responsiveness to exogenous ACTH (Sigma-Aldrich) *in vivo* was determined as previously described (Wang et al., 2014). Briefly, dexamethasone (Sigma-Aldrich) was injected i.p. at a dose of 5 μg/g at 18:00 PM and at 08:00 AM. Two hours later, the mice were anesthetized by ketamine (80 μg/g) and xylazine (10 μg/g). A femoral arterial catheter was placed using aseptic procedures before the collection of blood samples at 16:00–18:30 PM, and ACTH (30 μg/kg) was infused at 16:00–16:30 PM. Arterial samples (50 μl) were obtained immediately before and at 15, 60, and 120 min after ACTH administration. Each sample was replaced with an equal volume of saline-containing heparin (100 U/ml).

An *in vitro* ACTH stimulation test was performed in cultured Y1 cells, a murine adrenocortical tumor cell line. Y1 cells can express the ACTH receptor just as adrenocortical cells do. It is known that the first step of steroid synthesis is progesterone in many cells which synthesize steroids. Y1 cells can produce progesterone but not corticosterone because they do not express 21-hydroxylase enzyme (Davies and MacKenzie, 2003; Rainey et al., 2004). Progesterone production is dramatically increased in response to ACTH stimulation in Y1 cells. The production of progesterone in response to ACTH is therefore used to test adrenocortical responsiveness in Y1 cells (Koldzic-Zivanovic et al., 2006). After treatment of Y1 cells with LPS (10 ng/ml) in combination with or without GYY4137 (50 μM) for 24 h, the cells were stimulated with 10 ng/ml ACTH for another 6 h. The culture media were harvested to determine progesterone levels.

2.4. TdT-mediated dUTP nick-end labeling (TUNEL) assay

The adrenal glands were excised, fixed in 10% neutral formalin and embedded in paraffin. Then, the tissue blocks were cut into 5 μm-thick slides, and five slides were obtained from each tissue block. Cultured Y1 cells were fixed in 4% paraformaldehyde. Apoptotic cells in adrenal gland and apoptotic Y1 cells were detected by TUNEL technique using the One Step TUNEL Apoptosis Assay Kit (Beyotime, Jiangsu, China) according to the manufacturer's instructions. DAPI staining was used to determine the number of nuclei. The TUNEL signals were observed with fluorescence microscopy (Olympus, Japan). Cell apoptosis was determined as the ratio of the number of TUNEL-positive nuclei to that of Hoechst-positive nuclei. The results of relative fluorescence intensity of TUNEL were estimated by imageJ.

2.5. Mitochondria isolation

Mitochondria isolation was performed using the Mitochondria Fractionation Kit (Beyotime Inst. Biotech, Peking, PR China) as previously described (Wang et al., 2014). Briefly, adrenal glands

Please cite this article in press as: Wang, C., et al., Endogenous H_2S resists mitochondria-mediated apoptosis in the adrenal glands via ATP5A1 S-sulfhydration in male mice, Molecular and Cellular Endocrinology (2018), https://doi.org/10.1016/j.mce.2018.02.011