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Hydrogen sulfide modulates high glucose-induced NLRP3 inflammasome activation in 3T3-L1 adipocytes

TIAN-XIAO HU[1*], NING-NING ZHANG[2*], YUN RUAN[1], QING-YING TAN[1] and JING WANG[1]

[1]Department of Endocrinology, Chinese People's Liberation Army 903 Hospital, Hangzhou, Zhejiang 310013; [2]Department of Physiology, Naval Medical University, Shanghai 200433, P.R. China

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Abstract. Activation of the NACHT leucine rich repeat and pyd domains-containing 3 (NLRP3) inflammasome plays an important role in the initiation of inflammation in adipose tissue in diabetic patients. However, the mechanisms underlying this are not fully understood. Hydrogen sulfide (H_2S) has been shown to have anti-inflammatory properties in various cell types. The present study aimed to investigate the effect of H_2S on high glucose (HG)-induced NLRP3 inflammasome activation in adipocytes. Adipocytes were differentiated from 3T3-L1 cells and treated with low glucose (LG), HG, H_2S donor sodium hydrosulfide (NaHS) or N-acetyl-tyrosyl-valyl-alanyl-aspartyl chloromethyl ketone, an inhibitor of the cysteine protease caspase-1. The expression levels of NLRP3, apoptosis-associated speck-like protein containing A CARD (ASC) and caspase-1, and the release of interleukin (IL)-1β and IL-18 were measured. The results of the present study indicated that HG increased the expression levels of NLRP3, ASC and cleaved caspase-1, and the release of IL-1β and IL-18 in adipocytes. Caspase-1 inhibition abolished HG-induced production of IL-1β and IL-18 in adipocytes. Furthermore, NaHS inhibited the expression of NLRP3, ASC and cleaved caspase-1, and the production of IL-1β and IL-18 in adipocytes treated with HG. In conclusion, HG may increase and exogenous H_2S may inhibit HG-induced NLRP3 inflammasome activation in adipocytes.

Correspondence to: Dr Jing Wang or Dr Qing-Ying Tan, Department of Endocrinology, Chinese People's Liberation Army 903 Hospital, 14 Lingyin Road, Hangzhou, Zhejiang 310013, P.R. China
E-mail: wangjing200710@126.com
E-mail: tanqy1973@163.com

[*]Contributed equally

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Introduction

Type 2 diabetes mellitus (T2DM) presents a major and growing health problem throughout the world. Microvascular and macrovascular complications often occur in T2DM, and chronic low-grade inflammation plays a central role in this process (1-3). The inflammatory response is associated with both insulin resistance and the development of vascular complications in T2DM patients (4,5). T2DM patients with higher body mass index (BMI) and a large amount of adipose tissue are considered to be at greater risk of developing more severe insulin resistance (6,7) and cardiovascular disease (8,9).

Adipose tissue is regarded as an important endocrine organ and is known to be involved in regulating inflammation (10-12). Studies have shown that the NACHT leucine rich repeat and pyd domains-containing 3 (NLRP3) inflammasome plays a role in the initiation of inflammation in adipose tissue (3,13). A wide range of pathogens and cellular damage can activate the NLRP3 inflammasome, and its activation leads to caspase-1 activation and the release of the inflammatory cytokines interleukin (IL)-1 and IL-18 (14,15). It has been shown that the expression of NLRP3 is significantly elevated in adipose tissues of patients with obesity, dyslipidemia and diabetes, and is positively correlated with the severity of atherosclerosis (16). It is hypothesized that a high glucose (HG) environment may induce aberrant expression of the NLRP3 inflammasome in adipose tissue of T2DM patients, and that this may contribute to the development of atherosclerosis. However, the effects of HG on the expression of NLRP3 in adipocytes and adipose tissues remain to be elucidated.

Hydrogen sulfide (H_2S), a gaseous signaling transmitter, can be produced by a wide spectrum of mammalian tissues (17). Previous studies have indicated that H_2S plays numerous regulatory effects in the cardiovascular, gastrointestinal and neurological systems (18-21). The anti-inflammatory properties of H_2S have been identified in a range of cell types (22-24). In the cardiovascular system, H_2S is suggested to inhibit the development of atherosclerosis through suppression of inflammation (25,26). Cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS) are enzymes responsible for the synthesis of H_2S. It has been reported that both CSE and CBS are expressed in adipose tissues and adipocytes (27,28). Pan *et al* (29) demonstrated that HG inhibits expression of

CSE, and thus the production of H_2S, in adipocytes. This was confirmed by our previous study, which additionally revealed that HG did not affect the expression level of CBS (30). Several studies have also suggested that HG significantly increases the secretion of cytokines, including tumor necrosis factor-α (TNF-α), IL-6, monocyte chemoattractant protein-1 and adiponectin (31,32). However, the effect that this HG-induced reduction in CSE expression and H_2S production has on the expression of the NLRP3 inflammasome remains unknown. The hypothesis of the present study was that H_2S may play a role in HG regulation of NLRP3 expression in adipocytes. To test this hypothesis the expression of the NLRP3 inflammasome in adipocytes exposed to HG was investigated and an H_2S donor was used to try to reverse the HG effects.

Materials and methods

Cell culture and treatment. Adipocytes were cultured at 37˚C with 5% CO_2 and differentiated from 3T3-L1 cells (American Type Culture Collection) as previously described (30). After disassociation using 0.125% trypsin, $1x10^6$/ml adipocytes were seeded and grouped for treatment. To determine the effect of HG, cells were treated with either low glucose (LG) DMEM (5.5 mmol/l glucose; HyClone; GE Healthcare Life Sciences; cat. no. SH30021.01) or HG DMEM (25.0 mmol/l glucose; HyClone; GE Healthcare Life Sciences; cat. no. SH30022.01) for 24 h. To determine the effect of H_2S, cells were treated with HG DMEM containing increasing concentrations of sodium hydrosulfide (NaHS; Sigma-Aldrich; Merck KGaA; cat. no. 161527) or HG DMEM without NaHS for 24 h. Our previous study revealed that 10, 25 and 50 nmol/l are effective concentrations of NaHS for treatment of adipocytes, so those concentrations were used in the present study (30). To inhibit the activity of the NLRP3 inflammasome, cells were treated with HG DMEM containing 10 μg/ml N-acetyl-tyrosyl-valyl-alanyl-aspartyl chloromethyl ketone (Ac-YVAD-CMK); Sigma-Aldrich; Merck KGaA; cat. no. SML0429) or DMSO for vehicle for 24 h.

Western blot analysis. Proteins were extracted from adipocytes using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology). The protein concentration was assayed using a BCA Protein Assay kit (Beyotime Institute of Biotechnology). Protein samples (50 μg) were used for SDS-PAGE (4 and 10% gel) and subsequently transferred to nitrocellulose membranes. After blocking with 5% skim milk for 2 h at 37˚C, membranes were incubated with primary antibodies against NLRP3 (Abcam; cat. no. ab10931; 1:1,000), apoptosis-associated speck-like protein containing A CARD (CST Biological Reagents Co., Ltd.; cat. no. 4628; 1:1,000), caspase-1 (Santa Cruz Biotechnology, Inc.; cat. no. SC-514, 1:1,000) and β-actin (Sigma-Aldrich; Merck KGaA; cat. no. A5441; 1:8,000) for 12 h at 4˚C. The nitrocellulose membranes were then incubated with a secondary HRP-conjugated antibody (1:2,000). Immunoreactive proteins were then visualized using Immobilon Western Chemiluminescent HRP Substrate (Merck KGaA) and a Tanon 5200 Multi scanner (Tanon Science and Technology Co., Ltd.). The band intensities were calculated by ImageJ (version 1.51b; National Institutes of Health). Then the ratio of band intensities to β-actin was obtained to quantify the relative protein expression levels.

ELISA analysis of IL-1β and IL-18 release. After treatment, culture media were collected and the concentrations of IL-1β and IL-18 released into culture media were determined using IL-1β (cat. no. F10770) and IL-18 (cat. no. F10920) ELISA kits (Shanghai Westang Biotech Co., Ltd.) according to the manufacturer's instructions. All assays were performed in duplicate.

Statistical analysis. The data are presented as the mean ± SEM and were analyzed by one-way ANOVA followed by LSD-t test using SPSS (version 20; IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

HG significantly upregulates the expression of NLRP3 inflammasome in adipocytes. Activation of the NLRP3 inflammasome initiates inflammation in adipose tissues and adipocytes. To investigate whether HG is associated with activation of the NLRP3 inflammasome, its expression in adipocytes was observed. The expression levels of NLRP3 inflammasome components NLRP3, ASC and caspase-1 were determined by western blot analysis. Compared with the LG group, HG significantly increased the level of NLRP3 and ASC, and the ratio of cleaved caspase-1 to pro-caspase-1 (Fig. 1A-D).

HG stimulates the release of IL-1β and IL-18 by adipocytes via activation of the NLRP3 inflammasome. In the present study, mature IL-1β and IL-18 levels in culture media were determined by ELISA. The data revealed that IL-1β and IL-18 concentrations were significantly higher in the culture media of adipocytes treated with HG compared with those treated with LG. In order to confirm the role of NLRP3 inflammasome activation in this HG-induced IL-1β and IL-18 release, adipocytes were treated with Ac-YVAD-CMK. As shown in Fig. 2, the IL-1β and IL-18 concentrations in the culture media of adipocytes treated with HG + Ac-YVAD-CMK were significantly lower compared with HG DMEM containing vehicle.

Role of H_2S in the regulation of NLRP3 inflammasome activation in adipocytes. To confirm the role of H_2S in regulation of NLRP3 inflammasome activation, adipocytes were treated with HG DMEM containing increasing doses of the H_2S donor NaHS. The treatment of adipocytes with NaHS resulted in decreased HG-induced expression levels of NLRP3 and ASC, a reduced cleaved caspase-1 to pro caspae-1 ratio (Fig. 3), and a reduced HG-induced release of IL-1β and IL-18 (Fig. 4).

Discussion

The present study demonstrated that HG increased the activity of the NLRP3 inflammasome in adipocytes. Additionally, NaHS, an H_2S donor, inhibited HG-induced expression of the NLRP3 inflammasome and the release of IL-1 and IL-18.

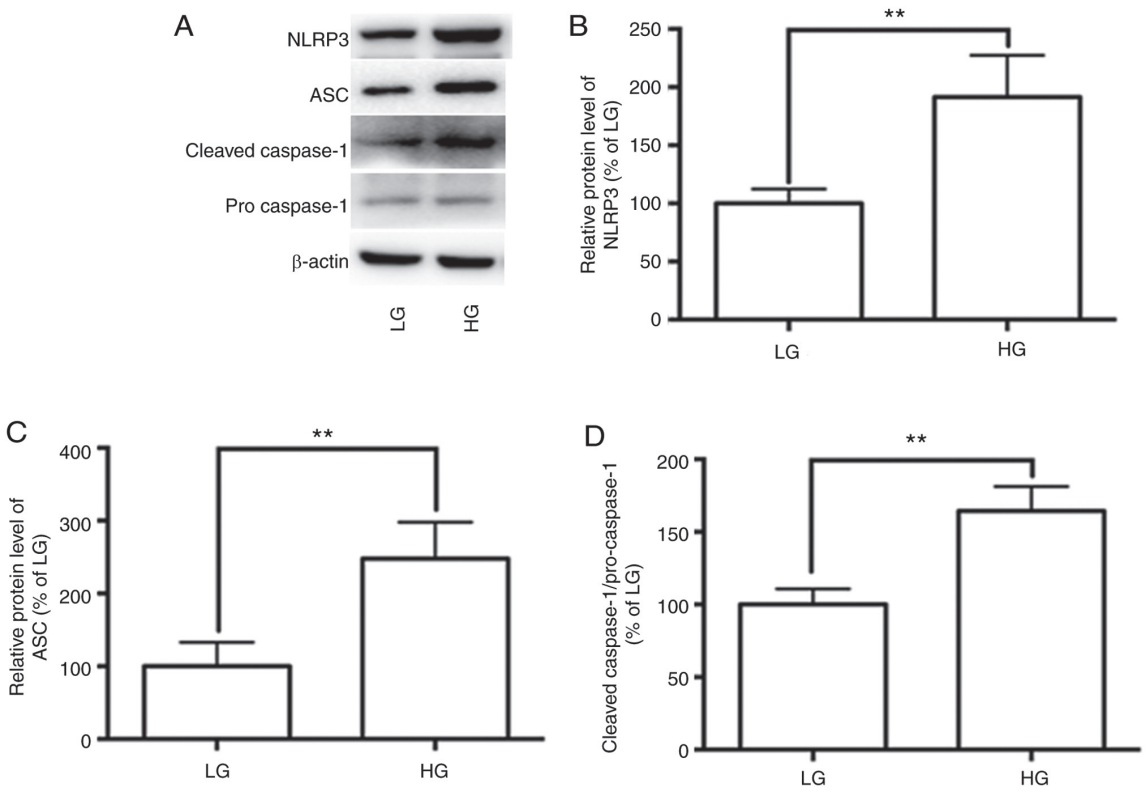

Figure 1. Effect of HG on the expression levels of NLRP3, ASC and caspase-1 in adipocytes. (A) Western blotting was performed to determine the expression levels of (B) NLRP3, (C) ASC and (D) the ratio of caspase-1 to pro caspase-1 in adipocytes treated with LG and HG. Data are presented as the mean ± SEM of four replicates (n=4 cultures). **P<0.01 as indicated. ASC, apoptosis-associated speck-like protein containing A CARD; HG, high glucose; LG, low glucose; NLRP3, NACHT leucine rich repeat and pyd domains-containing 3.

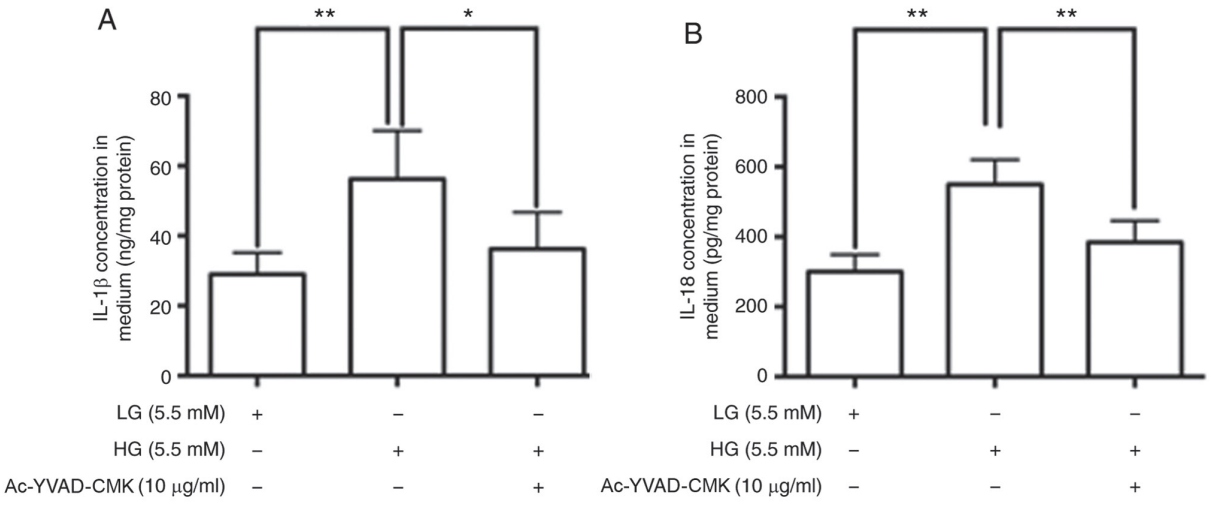

Figure 2. Effect of caspase-1 inhibitor Ac-YVAD-CMK on HG-induced IL-1β and IL-18 release in adipocytes. ELISA was performed to determine the concentrations of (A) IL-1β and (B) IL-18 in culture media from adipocytes treated with LG and HG. Data are presented as the mean ± SEM of four replicates. *P<0.05 and **P<0.01 as indicated. HG, high glucose; IL, interleukin; LG, low glucose; Ac-YVAD-CMK, N-acetyl-tyrosyl-valyl-alanyl-aspartyl chloromethyl ketone.

The link between T2DM and inflammation is well established. T2DM is considered to be, in part, a consequence of subclinical chronic low-grade inflammation (1-3). Several studies have reported that circulating inflammatory cytokines, such as c-reactive protein, TNF-α, IL-1β, IL-6 and IL-18, are significantly elevated in T2DM patients (33-38). Elevated levels of these inflammatory cytokines directly induce insulin resistance and impair glucose homeostasis (39-41). Cytokines of the IL-1 family are critical regulators of inflammation and control numerous inflammatory processes. Both IL-1β and IL-18, which are classic pro-inflammatory cytokines of the IL-1 family, contribute to insulin resistance and islet β-cell damage in T2DM (39,42,43). Additionally, chronic inflammation is also a major feature of atherosclerosis. In the progression of T2DM, IL-1β and IL-18 increase the risk of microvascular

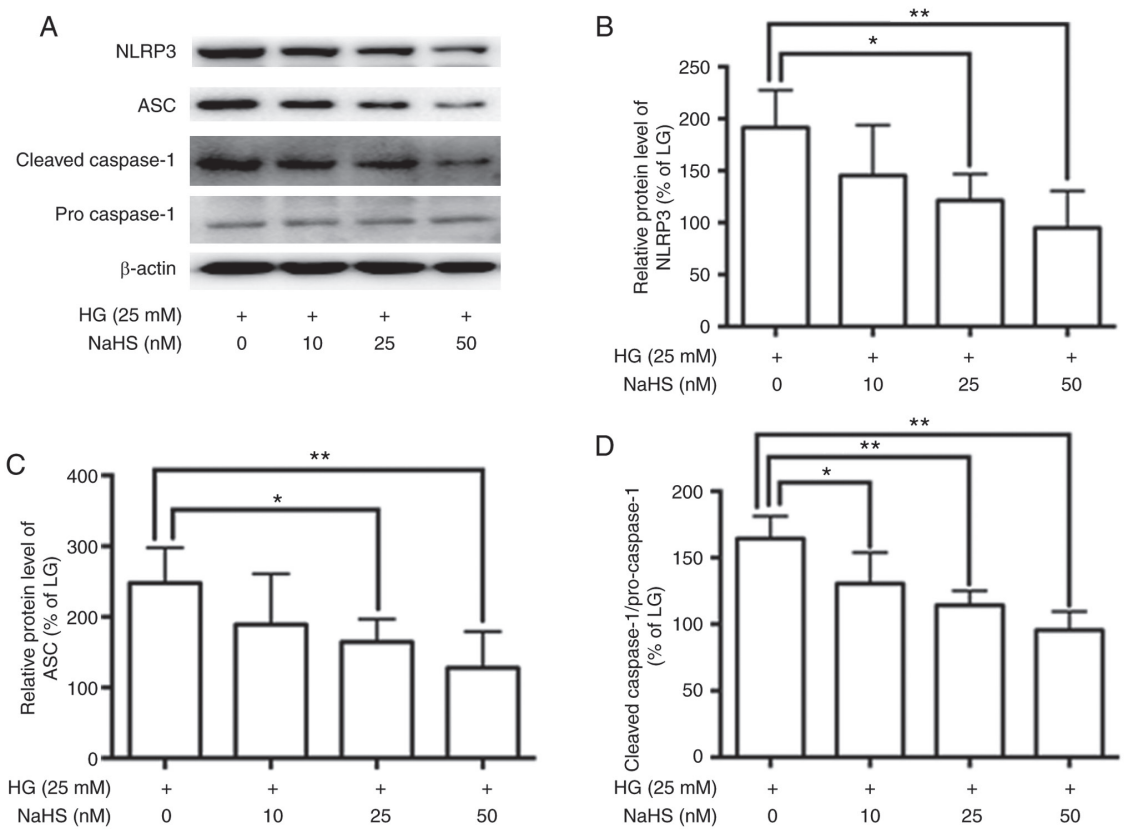

Figure 3. Effect of H_2S donor NaHS on HG-induced NLRP3 inflammasome expression in adipocytes. (A) Western blot analysis was performed to determine the expression levels of (B) NLRP3 and (C) ASC and (D) the ratio of caspase-1 to pro caspase-1 in adipocytes treated with HG DMEM containing increasing concentrations of NaHS (0, 10, 25, 50 nM). Data are presented as the mean ± SEM of four replicates. *P<0.05 and **P<0.01 as indicated. ASC, apoptosis-associated speck-like protein containing A CARD; H_2S, hydrogen sulfide; HG, high glucose; LG, low glucose; NaHS, sodium hydrosulfide; NLRP3, NACHT leucine rich repeat and pyd domains-containing 3.

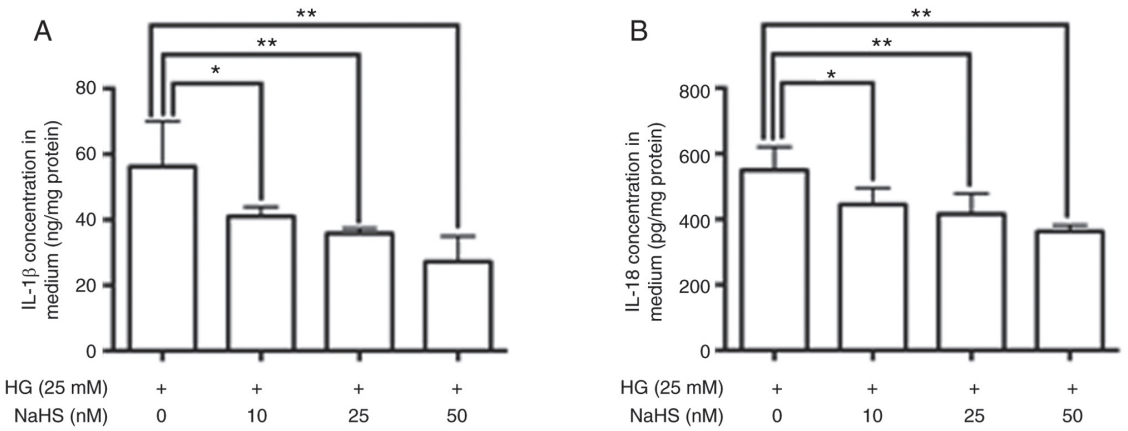

Figure 4. Effect of H_2S donor NaHS on HG-induced IL-1β and IL-18 release in adipocytes. ELISA was performed to determine the concentrations of (A) IL-1β and (B) IL-18 in culture media from adipocytes treated with HG DMEM containing increasing concentrations of NaHS (0, 10, 25, 50 nM). Data are presented as the mean ± SEM of four replicates. *P<0.05 and **P<0.01 as indicated. HG, high glucose; IL, interleukin; LG, low glucose; NaHS, sodium hydrosulfide.

and macrovascular complications by accelerating atherosclerosis (44). However, the underlying molecular mechanism behind the elevation of IL-1β and IL-18 levels in T2DM patients has not been fully elucidated.

The majority of cytokines in the IL-1 family have been linked to obesity (14,15). Accumulating evidence has indicated that the NLRP3 inflammasome plays a critical role in regulating IL-1β and IL-18 production in adipose tissues (3,13). Once NLRP3 is activated, the inflammasome recruits pro-caspase-1. The clustering of pro-caspase-1 subunits at the inflammasome complex results in auto-cleavage and formation of active caspase-1. Active caspase-1 converts pro-IL-1β and pro-IL-18 into their mature forms, IL-1β and IL-18. In the adipose tissues of obese individuals, compared with lean individuals, activity of the NLRP3 inflammasome and expression levels of IL-1β and IL-18 are significantly elevated (16,45,46).

T2DM patients with higher BMI and a greater amount of adipose tissue are found to have higher serum IL-1β and IL-18 levels, which results in more severe insulin resistance and increased risk of cardiovascular disease (35,47). Despite this, whether an HG environment effects NLRP3 inflammasome expression and IL-1β and IL-18 release in the adipose tissue of T2DM patients is unknown and merits further investigation. Results of the present study indicated that HG significantly increased the expression levels of NLRP3 and ASC, and caspase-1/pro capase-1 ratio in adipocytes. The results also suggested that HG significantly increased IL-1β and IL-18 release in the media of cultured adipocytes. In order to identify whether NLRP3 inflammasome activation was involved in HG-induced IL-1β and IL-18 release, cells were treated with HG DMEM containing an NLRP3 inflammasome inhibitor. Results indicated that inhibition of the NLRP3 inflammasome abolished HG-induced IL-1β and IL-18 release. These data suggest that HG increased the production of IL-1β and IL-18 in adipocytes via activation of the NLRP3 inflammasome.

H_2S, a gaseous signaling transmitter, is reportedly involved in inflammation in various tissues (17). Adipocytes have been shown to express both CBS and CSE, and the expression of CSE and the generation of H_2S have been shown to be suppressed by HG (29,48). As previously discussed, activation of the NLRP3 inflammasome and release of IL-1β and IL-18 increased in adipocytes exposed to HG. Therefore, it was hypothesized that a reduction in H_2S in HG DMEM may increase NLRP3 inflammasome expression and IL-1β and IL-18 release. In order to verify this hypothesis, adipocytes were treated with HG DMEM in the presence of increasing concentrations of H_2S donor NaHS. The findings indicated that NaHS significantly suppressed NLRP3 inflammasome expression and IL-1β and IL-18 release in adipocytes. These data suggest that exogenous H_2S can inhibit HG-induced NLRP3 inflammasome activation in adipocytes.

In summary, the results of the present study suggest that HG increased activation of the NLRP3 inflammasome in adipocytes. Exogenous H_2S donor NaHS significantly inhibited NLRP3 inflammasome expression, and IL-1β and IL-18 production in adipocytes.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TXH and NNZ were involved in drafting the manuscript. TXH, NNZ and YR collected and analyzed the data. QYT and JW interpreted the data, and all authors gave final approval of the version to be published. All authors reviewed the initial manuscript and revised it critically for important intellectual content.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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