Hydrogen sulphide reduces insulin secretion from HIT-T15 cells by a K_{ATP} channel-dependent pathway

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Abstract

Hydrogen sulphide (H_2S), a naturally occurring gas exerts physiological effects by opening K_{ATP} channels. Anti-diabetic drugs (e.g. glibenclamide) block K_{ATP} channels and abrogate H_2S-mediated physiological responses which suggest that H_2S may also regulate insulin secretion by pancreatic β-cells. To investigate this hypothesis, insulin-secreting (HIT-T15) cells were exposed to NaHS (100 μM) and the K_{ATP} channel-driven pathway of insulin secretion was tracked with various fluorescent probes. The concentration of insulin released from HIT-T15 cells decreased significantly after NaHS exposure and this effect was reversed by the addition of glibenclamide (10 μM). Cell viability and intracellular ATP and glutathione (GSH) levels remained unchanged, suggesting that changes in insulin secretion were not ATP linked or redox dependent. Through fluorescence imaging studies, it was found that K^+ efflux occurs in cells exposed to NaHS. The hyperpolarised cell membrane, a result of K^+ leaving the cell, prevents the opening of voltage-gated Ca^{2+} channels. This subsequently prevents Ca^{2+} influx and the release of insulin from HIT-T15 cells. This data suggest that H_2S reduces insulin secretion by a K_{ATP} channel-dependent pathway in HIT-T15 cells. This study reports the molecular mechanism by which H_2S reduces insulin secretion and provides further insight into a recent observation of increased pancreatic H_2S production in streptozotocin-diabetic rats.

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Introduction

Insulin is secreted mainly by pancreatic β-cells and it functions primarily to regulate glucose levels in the blood. β-Cells secrete insulin when circulating glucose levels are high by a mechanism that is largely driven by cellular ATP levels and K_{ATP} channels. When ATP levels are high in the β-cell, K_{ATP} channels in the cell membrane close causing cell membrane depolarisation and an increase in K^+ ion concentration within the cell. Membrane depolarisation thus results in the opening of voltage-gated Ca^{2+} channels leading to an influx of Ca^{2+} ions into the cell and the subsequent mobilisation and release of insulin-containing vesicles into the extracellular space (Cook *et al.* 1988, Ashcroft *et al.* 1989, Ashcroft & Gribble 1998). K_{ATP} channels are therefore crucial in this pathway since they act to link metabolic state of the cell with insulin secretion. Reduced insulin secretion and/or insulin sensitivity results in diabetes mellitus; a complex disease that is associated with marked changes in circulating glucose levels. Treatments for deficiencies of insulin secretion include modulators of K_{ATP} channel activity like the sulphonylureas (i.e. tolbutamide and glibenclamide) which close K_{ATP} channels and thus function as insulin secretagogues.

In recent years, much attention has been focused on hydrogen sulphide (H_2S) and its potential physiological effects in the cardiovascular system (Zhao *et al.* 2001). H_2S is endogenously produced from the amino acid, L-cysteine, by two key enzymes that are involved in the trans-sulphuration pathway, cystathionine-γ-lyase (CSE) and cystathionine-β-synthetase (CBS; Kamoun 2004). These two enzymes are distributed in a wide range of tissues, including the pancreas (Bhatia *et al.* 2005), and their expression and activities have been shown to be altered in a variety of pathophysiological conditions (Mok *et al.* 2004, Yan *et al.* 2004, Bhatia *et al.* 2005, Li *et al.* 2005). Human brain homogenates contain 50–160 μM H_2S (Goodwin *et al.* 1989) while substantial amounts (about 50–100 μM) are also found in human (Richardson *et al.* 2000) and rat (Zhao *et al.* 2001) serum. In experimental diabetes animal models, both streptozotocin and Zucker diabetic fatty rats had significantly higher H_2S formation in the pancreas (Jia *et al.* 2004, Yusuf *et al.* 2005). Increased CSE and CBS activity in diabetic animals and human have also been observed in different laboratories (Wijekoon *et al.* 2004, Herrmann *et al.* 2005). Plasma L-cysteine levels were elevated in diabetic patients with diabetic nephropathy renal complications (Herrmann *et al.* 2005). Elevated plasma homocysteine has also been reported in individuals with diseases of the metabolic syndrome including vascular disease and insulin resistance (Hayden & Tyagi 2004).

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Furthermore, endogenously produced H_2S has been shown to relax blood vessels via opening of vascular smooth muscle K_{ATP} channels; an effect attenuated by K_{ATP} channel antagonist, glibenclamide. Due to the importance of β-cell K_{ATP} channels in regulating insulin secretion and the reported effects of H_2S on vascular smooth muscle K_{ATP} channels, we considered whether H_2S acts as a putative, endogenous modulator of insulin secretion in pancreatic β-cells.

Recent studies show that homocysteine inhibited insulin secretion at both basal and stimulatory glucose levels from clonal BRIN-BD11 β-cells (Patterson *et al.* 2006*a*, 2007). Homocysteine also inhibited insulinotrophic responses to tolbutamide and KCl hinting of possible K-ATP channel involvement (Patterson *et al.* 2006*b*) in this inhibitory process. Endogenous CBS liberates H_2S from homocysteine. Since previous studies did not include inhibitors of CBS (such as addition of aminooxyacetic acid (AOAA)), it remains possible that H_2S generated from CBS contributes to the impaired release of insulin from clonal β-cells.

Therefore, in this current study, we aim to investigate the implications of H_2S exposure on β-cells (HIT-T15 cells). We also seek to determine the molecular changes in pancreatic β-cells after exposure to the H_2S donor, NaHS. We found that H_2S reduces insulin secretion in HIT-T15 cells without any effect on intracellular insulin. We provide evidence that the reduction in insulin secretion involves altered cell membrane K_{ATP} channel activity which triggers a cascade of molecular events that ultimately lead to altered insulin secretion from HIT-T15 cells. This study shows that H_2S is not merely a bystander molecule under the pathophysiological conditions of diabetes for it can play a primary role by further inhibiting insulin secretion from pancreatic β-cells.

Material and Methods

Cell culture

Syrian hamster pancreatic β-cells were obtained from American Type Culture Collection (HIT-T15) and cultured in HAMS F12K media with 1% (v/v) penicillin/streptomycin, 10% (w/v) horse serum (Sigma) and 2·5% (w/v) foetal bovine serum (Hyclone, South Logen, UT, USA), and 5% CO_2/95% O_2 with 95% humidity to an approximate 70% confluency before use. These cells secrete insulin in the presence of glucose in a K_{ATP} channel and Ca^{2+} channel activity-dependent pathway (He *et al.* 2003). Cells were lysed in phosphate buffer containing protease inhibitor cocktail mix (Sigma) and subjected to five freeze thaw cycles in liquid N_2 followed by 37 °C thawing. To ensure complete rupture of cells, lysates were further pulsed in a sonicator (Misonix Inc., Farmingdale, NY, USA) at a frequency of 10 kHz for 15 s.

Insulin assay

Cellular/media insulin was assayed by ELISA in accordance with the manufacturer's instructions (Crystal Chem. Inc., Downers Grove, IL, USA). Briefly, wells were coated with cell lysate/media (5 μl) and guinea pig anti-insulin (50 μl) for 18 h, rinsed with washing buffer (PBS containing 0·04% (v/v) Tween 20) and incubated with anti-guinea pig antibody enzyme conjugate (100 μl) for 3 h. After thorough rinsing, wells were incubated with enzyme substrate solution (100 μl) and the reaction stopped 30 min later by the addition of sulphuric acid (1 M, 50 μl). Absorbance (measuring wavelength, 492 nm; subtracting wavelength, 630 nm) was determined 10 min thereafter (Tecan Instruments Inc., Durham, NC, USA). Cell/media insulin concentration was obtained by comparing absorbances with an intra-plate rat insulin standard curve of concentrations (0–10 ng/ml). All samples were assayed in duplicate and results show insulin concentration in mg/ml.

Cellular ATP, glutathione (GSH) and cell viability assay

Intracellular ATP and GSH were assayed as described previously (Whiteman *et al.* 2003). Briefly, cells were seeded in 24-well plates at a density of $0{\cdot}2\times10^6$ cells per well. After treatment, cells were washed once with ice-cold PBS. ice-cold trichloroacetic acid (200 μl; 6·5% (w/v)) was then added into each well to cause protein precipitation. Cellular ATP was assessed using firefly lantern extract. Sample (3 μl) was incubated with 200 μl sodium arsenite buffer (comprising 26·67 mM $MgSO_4{\cdot}7H_2O$/3·33 mM KH_2PO_4/33·33 mM $Na_2HASO_4{\cdot}7H_2O$ (pH 7·4)). After the addition of 10 μl filtered firefly lantern extract per sample, light emission was then measured for 10 sec per sample using a LUMI-ONE portable luminometer (Trans Orchid Enterprises, Tampa, FL, USA). Concentrations of ATP were then determined by comparing the values obtained with a freshly prepared standard curve of ATP (0–40 nmol/ml). Cellular GSH was assessed using *o*-phthalaldehyde (OPT). Trichloroacetic acid (7·5 μl) extract was added to 96-well fluorescence plates followed by the addition of 227·5 μl of 100 mM KH_2PO_4–KOH buffer (pH 10·0) and 15 μl *o*-phthaldialdehyde (10 mg/ml freshly prepared in methanol). Samples were stored in the dark at room temperature for 25 min and measured by fluorescence (excitation = 350 nm, emission = 420 nm) using a Gemini Fluorescence plate reader (Molecular Devices). Concentrations of GSH were then determined by comparing the values obtained with a freshly prepared standard curve of GSH (0–30 nmol/ml). Cell viability was determined using the 3-(4, 5-dimethyl-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction assay (Mosmann 1983). Briefly, cells treated in 96-well plates were washed with PBS. Culture medium (200 μl) containing 0·5 mg/ml dissolved MTT was added to each well and incubated at 37 °C in the dark for 1 h. Cells were washed once with PBS and 200 μl DMSO were added to solubilise the formazan dye. Absorbance at 550 nm was then read on Molecular Devices SpectraMax190 plate reader after gentle shaking in the dark for 10 min.

Cell transfection and immunoblotting

Total RNAs were isolated from rat hippocampus and reverse transcribed using standard molecular biology methods. CBS cDNA was amplified using *CBS* gene-specific primers (sense 5′-ATCTGACTCGAGG*CCACC*ATGCCTTCAGGGA-CA-3′ and anti-sense 5′-ATTGCGGCCGCTTACTATT-TCCGGGTCTGCTC-3′) that incorporated XhoI and NotI sequences to facilitate cloning into pCIneo vector (Promega). pCIneo vector harbouring CBS full cDNAs were verified by restriction enzymes and DNA sequencing. CBS plasmid (4 μg) was mixed with 4 μl Lipofectamine 2000 (Invitrogen) in HAMS F12K media for 20 min prior to cell addition. Transfection media were replaced after 6 h with complete HAMS F12K media for 12 h. Cells were subsequently lysed with RIPA buffer containing protease inhibitor cocktail mixture (Sigma) and 20 μg lysates were loaded into each well for SDS-PAGE (12% acrylamide) and subsequent western transfer. Membranes were probed with CBS polyclonal antibodies (1: 4000; Abnova, Taipei City, Taiwan, ROC) overnight. After incubation, the membrane was washed and exposed to horseradish peroxidase (HRP)-conjugated secondary antibodies (1: 1000; BD Pharmingen, San Jose, CA, USA) for 1 h. The respective protein bands were then visualised by chemiluminescence using the SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA) system on an SR-2000 chemiluminescence image station (Kodak).

H_2S synthesis assay

Cells were lysed (rapid freeze/thawing) in phosphate buffer containing protease inhibitors. H_2S synthesis assay was carried out using the *N,N*-dimethyl-*p*-phenylenediamine sulphate (NNDPD) method as described previously (Mok *et al.* 2004, Li *et al.* 2005, Yusuf *et al.* 2005). Briefly, the assay mixture (500 μl) contained cell lysate (430 ml), L-cysteine (10 mmol/l; 20 μl, CBS substrate), pyridoxal 5′-phosphate (2 mmol/l; 20 μl; CBS cofactor) and saline (30 μl). After incubation (37 °C, 30 min), zinc acetate (1% (w/v), 250 μl) was injected to trap generated H_2S followed by trichloroacetic acid (10% (w/v), 250 μl) to precipitate protein and thus stop the reaction. Subsequently, (NNDPD, 20 mmol/l; 133 μl) in 7·2 mol/l HCl was added followed by $FeCl_3$ (30 mmol/l; 133 μl) in 1·2 mol/l HCl and absorbance (670 nm) of aliquots of the resulting solution (300 μl) was determined. The H_2S concentration of each sample was calculated against a calibration curve of NaHS (0–250 nmol/ml).

K^+, Ca^{2+} and membrane polarity determination

For measurements of K^+, Ca^{2+} and membrane polarity, cells grown in 96-well plates till confluent. For intracellular K^+ determination, cells were pre-loaded with K^+ fluorescent probe PBFI-AM (Molecular Probes, Carlsbad, CA, USA; 5 μM) in serum-free HAMS F12K media containing pluronic acid F-127 (0·05%) for 40 min at 37 °C (Bortner *et al.* 1991). For intracellular Ca^{2+} determination, cells were pre-loaded with Ca^{2+}-specific fluorescent probe Fluo-3-AM (Molecular Probes; 2 μM) in serum-free HAMS F12K media containing the detergent pluronic acid F-127 (0·05%) for 1 h at 37 °C (Wang *et al.* 2002). For membrane polarity determination, cells were pre-loaded with fluorescent probe DisBAC$(2)_3$ (1 μM final concentration in serum-free HAMS F12K media) for 20 min at 37 °C (Bouchot *et al.* 2001). After the respective incubation periods, cells were washed once with PBS and treated with various chemicals in serum-containing HAMS F12K media. Upon treatment, the 96-well plates were immediately incubated in a fluorescence plate reader (Molecular Devices), and changes in fluorescent intensity were determined after 5 min at wavelengths Ex340, 350 nm/Em500 nm (PBFI-AM), Em506 nm/Ex526 nm (Fluo-3-AM) and Em535 nm/Ex560 nm (DisBAC$(2)_3$). The fluorescence intensity data generated were then expressed as percentage change over the fluorescence intensity of the untreated control.

Statistical analysis

Data show mean ± S.E.M. Statistical analysis of data was by one-way ANOVA followed by *post hoc* Tukey's test. A P value of $<0{\cdot}05$ was taken to indicate a statistically significant difference.

Results

Effect of NaHS on cell viability, ATP, GSH and intracellular insulin

NaHS (100 μM) had no significant effect on cell viability and intracellular insulin in HIT-T15 cells (Table 1). Cells were exposed to various concentrations of NaHS and no significant change in cell viability was detected at concentrations of up to 100 μM, as assessed using the MTT reduction assay 24 h after

Table 1 Effect of 12 h NaHS (100 μM) treatment on HIT-T15 intracellular ATP, intracellular glutathione (GSH), cell viability, intracellular insulin and extracellular insulin (secreted into media). Data show mean ± S.E.M.

	Untreated control	NaHS treated
Cellular ATP concentration (μmol/mg protein)	26·5 ± 1·7	31·1 ± 2·6
Cellular GSH concentration (μmol/mg protein)	25·9 ± 1·8	25·5 ± 2·5
Cell viability (per cent of untreated control)	100·0 ± 2·9	96·4 ± 2·5
Amount of cellular insulin (ng/mg cell protein)	4·9 ± 0·3	4·3 ± 0·3
Insulin secreted (ng/mg cell protein)	1·3 ± 0·1	0·5 ± 0·1**

$n=6$, **$P<0{\cdot}01$ (cf. untreated control).

exposure (98·1±4·6% NaHS compared with 100±5·9% control). After 12 h, NaHS (100 μM) did not significantly alter HIT-T15 cellular levels of ATP or reduced (GSH; Table 1). Thus, data from the cell viability assay as well as the intracellular ATP and GSH assay suggest that NaHS (100 μM) did not compromise cellular ATP and GSH concentrations in HIT-T15 cells. Intracellular insulin levels were also determined in cells exposed to NaHS (100 μM) to verify that insulin content was not significantly altered (Table 1).

Effect of NaHS on insulin secretion

HIT-T15 cells secrete insulin in the presence of HAMS F12K media that already contain optimal concentrations of glucose (Muller *et al.* 1992). After 12 h, untreated HIT-T15 cells secreted 1·3±0·1 ng/mg cell protein ($n=6$), into the media. Culturing cells in the presence of dexamethasone significantly reduced insulin secretion by HIT-T15 cells to 0·4±0·1 ng/mg cell protein ($n=6$, $P<0·01$). Dexamethasone is known to inhibit insulin secretion through a genomic action in β-cells that leads to a decrease in the efficacy of cytoplasmic Ca^{2+} in the exocytotic process (Lambillotte *et al.* 1997). Intriguingly, a significant reduction in insulin secreted by HIT-T15 cells exposed to NaHS (100 μM) for 12 h was observed. This effect of NaHS was abolished in the presence of glibenclamide (K_{ATP} channel antagonist, 10 μM; Fig. 1), suggesting that NaHS-induced inhibition of insulin release occurs via a mechanism which involved the opening of K_{ATP} channels.

Changes in cellular K^+, Ca^{2+} and cell membrane polarity in the presence of NaHS

In an attempt to uncover the mechanism underlying the ability of NaHS to inhibit insulin secretion and also to verify the involvement of K_{ATP} channels, we carried out additional experiments to trace changes in several biological ions in HIT-T15 cells exposed to NaHS using ion-specific fluorescent probes. These probes were loaded in the cells prior to treatment and changes in fluorescence determined after NaHS exposure (Fig. 2a). The K^+ ion fluorescent probe

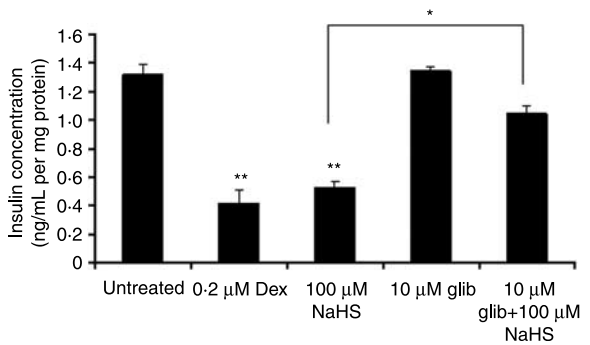

Figure 1 Insulin concentration in media of HIT-T15 cells treated with various drugs for 12 h. Data show mean±S.E.M., $n=4$–6, $^*P<0·05$, $^{**}P<0·01$ (cf. untreated cells).

PBFI-AM was used to detect changes in intracellular K^+ ions. Figure 2b shows that NaHS lowered intracellular K^+ ion levels in HIT-T15 cells, suggesting that NaHS caused K^+ channel opening in these cells. This effect was reversed by glibenclamide (10 μM) providing further evidence that NaHS most likely acts to open K_{ATP} channels to trigger K^+ ion efflux. The reduction in intracellular K^+ ion concentration caused by NaHS was greater than that caused by valinomycin (1 μM) which is a K^+-specific ionophore. Valinomycin is known to carry K^+ ions from the mitochondria to the cytosol thereby increasing the intracellular K^+ ion concentration (Benz *et al.* 1973) which might explain the observed greater K^+ ion concentration in cells exposed to valinomycin, as compared with cells exposed to NaHS. In contrast, there was, however, no significant difference in K^+ efflux in HIT-T15 cells exposed to cromakalim (100 μM). Cromakalim is a benzopyran K_{ATP} channel opener that has been shown to act on both sarcolemmal and mitochondrial K_{ATP} channels (Inoue *et al.* 1991, Paucek *et al.* 1995, Garlid *et al.* 1996). Thus, similar to valinomycin, cromakalim may cause the movement of K^+ ions from mitochondria to the cytosol. Since the PBFI-AM probe is localised to the cytosol of HIT-T15 cells, the opening of both cell membrane and mitochondrial K_{ATP} channels by cromakalim possibly results in no net change in cytosolic K^+ ion concentration, as observed in Fig. 2b. Figure 2c shows a decrease in the signal for the cell polarity fluorescent probe $DisBAC(2)_3$ from cells that were exposed to NaHS (100 μM). The percentage of $DisBAC(2)_3$ fluorescence in HIT-T15 cells exposed to NaHS was only 68·3±0·3% ($n=4$, $P<0·05$) of that of the untreated cells. This suggests that the HIT-T15 cell membrane is now more hyperpolarised because an increase in $DisBAC(2)_3$ fluorescence within cells commonly denotes cell membrane depolarisation. Intracellular Ca^{2+} ion levels were also determined in cells exposed to NaHS using Fluo-3-AM, a Ca^{2+}-specific intracellular fluorescent probe. Figure 2d shows that intracellular Ca^{2+} ion concentration was significantly lower (79·9±4·1%, $n=8$, $P<0·05$) in cells exposed to NaHS (100 μM) as compared with the untreated cells (100·0±6·7%, $n=6$). The NaHS-induced decrease in intracellular Ca^{2+} ion concentration was significantly reversed (108·8±5·8%, $n=8$, $P<0·05$) by glibenclamide (10 μM). Thapsigargin (1 μM), an inhibitor of sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps (Lytton *et al.* 1991), was used as a positive control for the Fluo-3-AM Ca^{2+}-sensitive assay.

CBS overexpression and its effect on insulin secretion in HIT-T15 cells

In an attempt to examine the effect of endogenous H_2S altering insulin release, HIT-T15 cells were transfected with an expression vector harbouring the *CBS* gene. CBS overexpression in HIT-T15 cells was performed as an earlier study found increased expression of CBS in the pancreas of the STZ-diabetic rat (Yusuf *et al.* 2005). HIT-T15 cells were

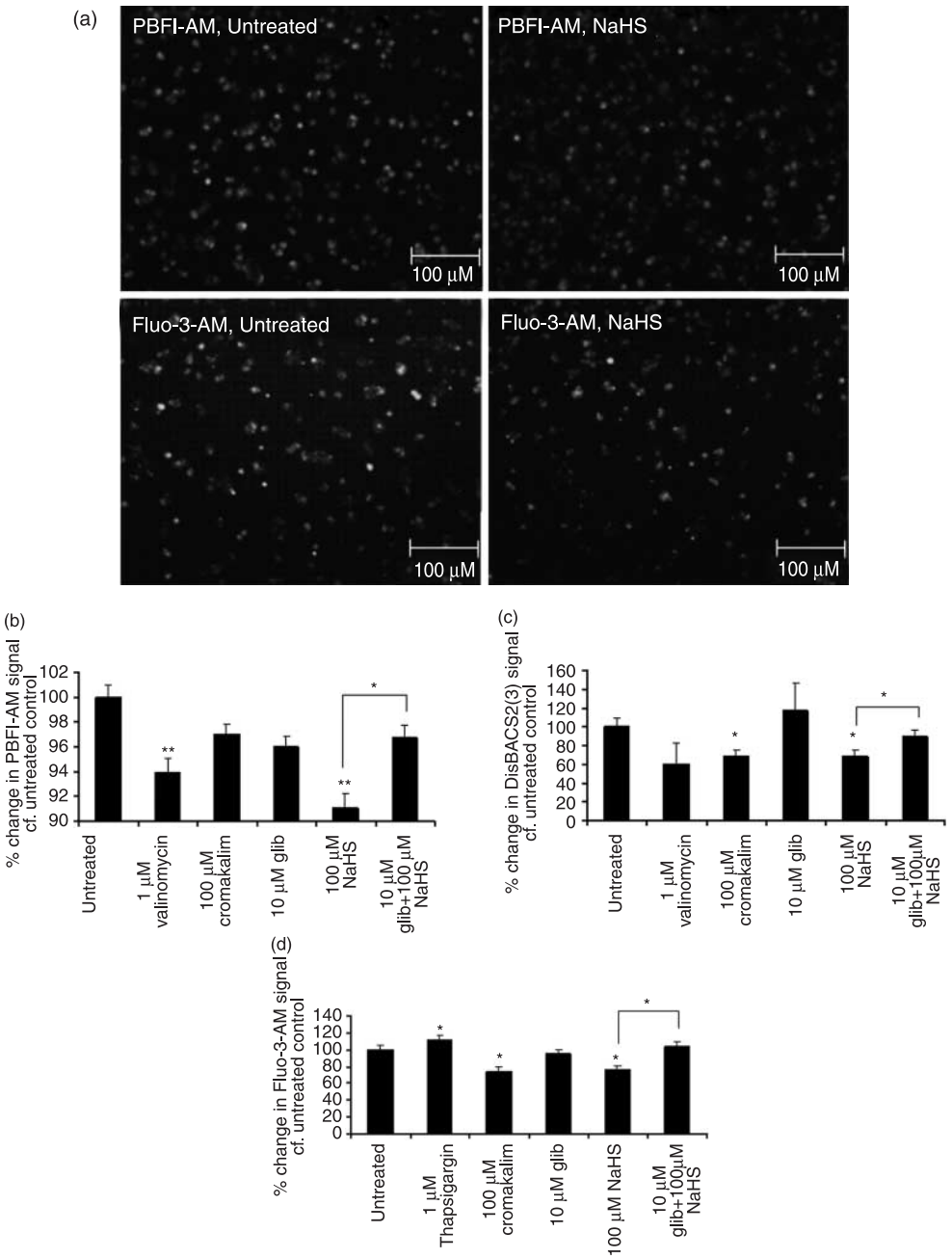

Figure 2 HIT-T15 cells pre-loaded with PBFI-AM (a, top two images) and Fluo-3-AM (a, bottom two images). Reduced PBFI-AM fluorescence (b) and DisBAC(2)$_3$ at (c) and Fluo-3-AM (d) in HIT-T15 cells show that NaHS (100 μM) treatment causes K^+ efflux and plasma membrane hyperpolarisation and lower intracellular Ca^{2+} concentrations respectively. Change in fluorescence intensity was calculated 5 min after treatment and data generated were then expressed as percentage change over the fluorescence intensity of the untreated control. Results show mean±S.E.M., n=4–8, *P<0·05, **P<0·01 (cf. untreated cells).

transfected with the *CBS* transcript gene for 12 h after which immunoblotting of the transfected cell lysates showed that there was an almost tenfold increase in CBS protein detected (Fig. 3a). However, western blot detection of CBS protein is not sufficient to determine CBS functionality and hence it became necessary to test for the increase in CBS enzymatic activity in the transfected cells. Cell lysates were exposed to high cysteine (CBS substrate) and pyridoxal-5-phosphate

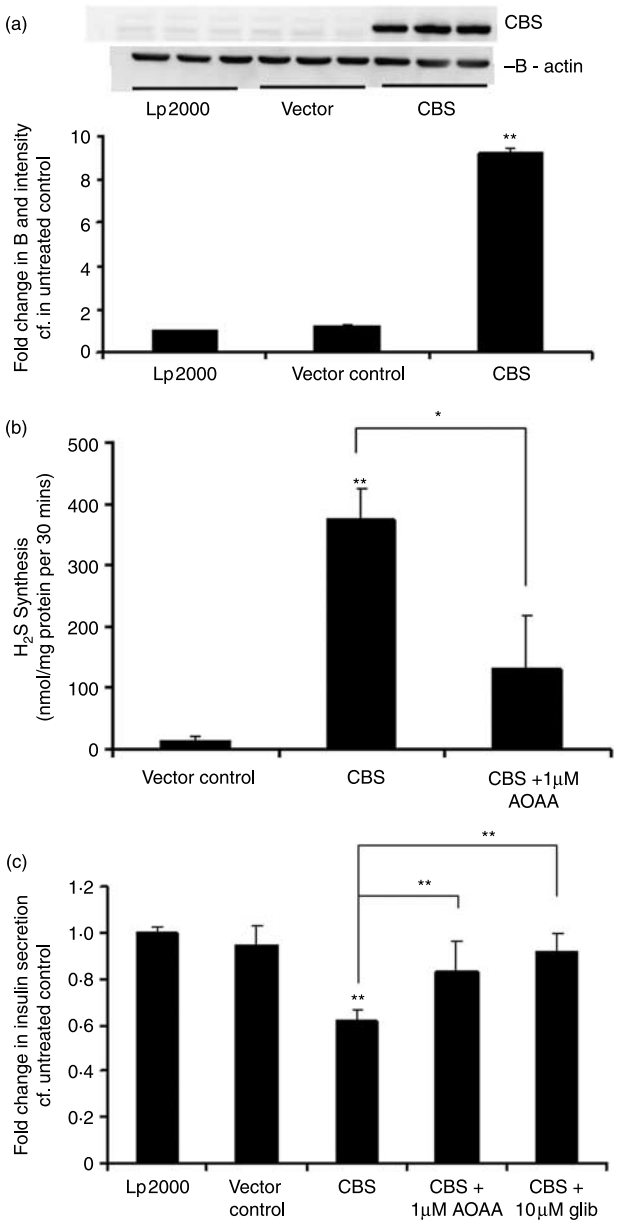

Figure 3 Overexpression of the *CBS* gene in HIT-T15 cells. (a) Western blots showing the increased amount of absolute CBS protein 24 h after transfection. (b) HIT-T15 cells transfected with CBS synthesised higher levels of H_2S, proving CBS functionality. (c) Overexpression of CBS decreased insulin secretion from HIT-T15 cells. Addition of 1 mM AOAA and 10 μM glibenclamide reversed the reduced insulin secretion seen in HIT-T15 cells overexpressing CBS. Lp2000 represents vehicle Lipofectamine 2000 treatment, vector control represents pCI-neo vector without *CBS* gene insertion. Results show mean ± S.E.M., $n=4$–8, $^*P<0{\cdot}05$, $^{**}P<0{\cdot}01$ (cf. untreated control).

(CBS cofactor) concentrations so as to determine activity of H_2S-producing enzymes. Figure 3b shows a 40-fold increase in H_2S synthesis in cells that were transfected with the CBS plasmid when compared with cells that were transfected with the vector alone, indicating that the transfection did indeed increase H_2S synthesis in HIT-T15 cells. The AOAA, an inhibitor of CBS, lowered the concentration of H_2S generated, from $374{\cdot}8 \pm 52{\cdot}1$ to $131{\cdot}3 \pm 82{\cdot}1$ nmol/mg protein ($n=4$, $P<0{\cdot}05$), strongly suggesting that the CBS transfection did result in an overproduction of a functional CBS protein. After verifying CBS protein functionality in CBS-transfected HIT-T15 cells, the concentration of insulin secreted into the media was measured to determine whether CBS overexpression affected insulin secretion. Insulin secretion from HIT-T15 cells overexpressing the CBS protein decreased to $61{\cdot}9 \pm 4{\cdot}4\%$ ($n=8$, $P<0{\cdot}01$) of that of the vector-alone transfected cells as seen in Fig. 3c. The addition of 1 mM AOAA reversed the reduction of insulin secretion seen in cells overexpressing CBS strongly suggesting that CBS activity decreases insulin secretion in HIT-T15 cells. The addition of 10 μM glibenclamide to transfected cells significantly attenuated CBS-induced impairment from $61{\cdot}9 \pm 4{\cdot}4$ to $91{\cdot}5 \pm 8{\cdot}4\%$ ($n=4$–8, $P<0{\cdot}05$) of that of vector-alone transfected cells (Fig. 3c). This confirms the crucial role of K-ATP channels in H_2S- and CBS-induced reduction in insulin secretion. There was no change in total intracellular insulin content between CBS-transfected ($5{\cdot}2 \pm 0{\cdot}1$ ng/mg cell protein) and vector-transfected cells ($5{\cdot}0 \pm 0{\cdot}2$ ng/mg cell protein, $n=4$, $P>0{\cdot}05$). There was also no change in cell viability between CBS-transfected ($96{\cdot}4 \pm 2{\cdot}5\%$ of untreated cells) and vector-transfected cells ($101{\cdot}7 \pm 0{\cdot}7\%$ of untreated cells, $n=4$, $P>0{\cdot}05$).

Discussion

We have previously shown a marked increased H_2S synthesis in liver and pancreas from streptozotocin-induced diabetic rats (Yusuf *et al.* 2005). We further showed that such increase in H_2S synthesis was most likely a result of increased CSE (liver only) and CBS (liver and pancreas) expression.

In order to further understand the part played by H_2S in glucose homeostasis, we evaluated the effect of NaHS (H_2S donor) on HIT-T15 cell metabolism and insulin secretion. HIT-T15 cells are insulinoma pancreatic β-cells derived from the islet cells of mammalian Syrian hamster and transformed by the simian virus 40. Though insulinomas, these cells retain most of the differentiated functions characteristic of β-cells and, as such, provide a widely used model system for studying the regulation of this endocrine cell (Santerre *et al.* 1981). It should be noted that since only ~30% of NaHS in solution is present as free H_2S gas (Zhao & Wang 2002), cells subjected to 100 μM NaHS in the present experiments were exposed to 30 μM free gas, a concentration which is well within the reported physiological concentrations of H_2S in, for example, mammalian blood and tissues (Richardson *et al.* 2000, Zhong *et al.* 2003, Mok *et al.* 2004).

We found that exposure of HIT-T15 cells to NaHS did not significantly change cell viability, intracellular ATP or intracellular GSH concentrations. These data indicate that

under the experimental conditions employed, HIT-T15 cells exposed to 100 μM NaHS (i.e. 30 μM H_2S gas) do not appear to undergo either respiratory chain inhibition or oxidative stress. Insulin secretion though was impaired in HIT-T15 cells exposed to optimal glucose levels in the media in the presence of NaHS even though the cellular concentration of insulin remained unchanged. HIT-T15 cells have a high constitutive release rate of insulin with optimal concentrations of glucose (already present in the culture media); however, the relative stimulatory response to increased glucose levels is low. This might explain why glibenclamide alone had no stimulatory effect on insulin secretion. However, glibenclamide did reverse the inhibition of insulin release caused by NaHS, suggesting that NaHS acts through K_{ATP} channels.

Our data confirm a previous report which demonstrated that NaHS inhibits insulin secretion via K_{ATP} channels albeit in a different insulinoma cell line (Yang *et al.* 2005). A similar observation has also recently been made using mouse primary islet cultures (Kaneko *et al.* 2006). However, this is the first study which has attempted to identify the underlying cellular mechanism(s) by which NaHS inhibits insulin release from HIT-T15 cells. Our findings suggest that the inhibition of insulin release caused by NaHS is driven primarily by the opening of K_{ATP} channels as evidenced by the loss of intracellular K^+ ions and the ability of glibenclamide to reverse the NaHS-induced inhibition of insulin release. The concomitant opening of K_{ATP} channels results in a loss of intracellular K^+ ions, as seen with lower PBFI fluorescence. Cell membrane hyperpolarisation ensues as a probable result of cellular K^+ ion loss for decreased cell fluorescence of $DisBAC(2)_3$ was observed in HIT-T15 cells treated with NaHS. In pancreatic β-cells, membrane depolarisation drives the opening of voltage-gated Ca^{2+} channels leading to an influx of Ca^{2+} ions and a subsequent release of insulin from the cell. When HIT-T15 cells were exposed to NaHS, the membrane was observed to become more hyperpolarised and as a result of a change in state of voltage-gated Ca^{2+} channels, less Ca^{2+} was present in the cytosol of HIT-T15 cells. This probably explains why a significant decrease in intracellular Ca^{2+} was detected when using the Ca^{2+}-specific Fluo-3 AM probe. The addition of glibenclamide significantly reversed both the NaHS-induced loss of $DisBAC(2)_3$ and Fluo-3-AM signal from HIT-T15 cells. This shows that the NaHS-induced change in K-ATP channel activity is responsible for membrane hyperpolarisation and NaHS-induced Ca^{2+} efflux in HIT-T15 cells. This is the first report that proposes a mechanism by which NaHS reduces insulin secretion from β-cells via modulation of K-ATP channels.

The significance of these finding goes beyond the main observation that H_2S inhibits insulin secretion. First, HIT-T15 cells express low levels of CBS with no CSE mRNA transcripts found (data not shown). By overexpressing CBS in HIT-T15 cells, the resultant functional CBS protein synthesises H_2S from L-cysteine causing a decrease in insulin release even in the presence of glucose. Both AOAA (inhibitor of CBS) and glibenclamide attenuated the CBS protein-induced reduction in insulin secretion from HIT-T15 cells. This suggests that the CBS protein indirectly regulates insulin release from HIT-T15 cells via modulation of K-ATP channel activity. Secondly, CBS expression has been reported to increase in the pancreas of the streptozotocin-diabetic rat (Yusuf *et al.* 2005). An increase in pancreatic CBS expression would be expected lead to an increase in H_2S secretion in the pancreas which might directly reduce insulin secretion by opening K_{ATP} channels in β-islet cells. Therefore, an increase in CBS expression in the pancreas may possibly exacerbate the diabetic condition in the streptozotocin-diabetic rat. Thirdly, diabetic patients were found to have elevated homocysteine levels (Hayden & Tyagi 2004). In the presence of endogenous CBS, elevated homocysteine levels may cause H_2S levels to be elevated in diabetic patients. Homocysteine has recently been shown to impair insulin secretion in clonal β-cells. This study suggests that the H_2S probably contributes to the impairment of insulin secretion induced by homocysteine. Hence, elevated homocysteine levels in diabetic patients can impair insulin secretion from β-cells and exacerbate the diabetic condition through the generation of H_2S.

Numerous biologically active gases, namely NO, CO and H_2S have been the subject of scientific interest in recent years. It was recently shown that exogenous NO inhibits, while CO increases glucose-stimulated insulin secretion in intact mouse islets (Mosen *et al.* 2006). Here, we show that both exogenous (NaHS) and endogenous H_2S reduce insulin secretion from HIT-T15 cells through a mechanism involving K-ATP channels. We suggest that greater emphasis should be placed on such gases and their respective cellular mechanism(s) when working on physiological/pathophysiological conditions which effect insulin release.

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