ORIGINAL ARTICLE ASIAN JOURNAL OF MEDICAL SCIENCES

Increased H_2S synthesizing activity in plasma correlated to fasting blood glucose levels: A study in type 2 diabetes patients

Pinaki Saha[1], Sayantan Dasgupta[2], Sukla Nath[2], Pradipta Ghosh[3], Santanu Sen[4], Tanmay Jyoti Sau[5], Arun Kumar[6], Utpal Kumar Biswas[7]

[1]Junior Resident, [2]Assistant Professor, [3]Demonstrator, Department of Biochemistry, Nilratan Sircar Medical College, [4]Associate Professor, Department of Biochemistry, Calcutta National Medical College, [5]Professor, Department of General Medicine, Calcutta Medical College, [6]Professor, Department of Biochemistry, Gouri Devi Institute of Medical Sciences, Durgapur, [7]Professor, Department of Biochemistry, Institute of Post Graduate Medical Education and Research, Kolkata, West Bengal, India

Submitted: 05-07-2016 Revised: 26-08-2016 Published: 01-11-2016

ABSTRACT

Background: Diabetes mellitus is a major health problem not only in India but worldwide. Our country presently is undergoing an epidemic stage of this non-communicable disease. Though several etiological background of type 2 diabetes has been well explained, yet a number of recent literatures suggested a potential role H_2S producing enzymes in the etiology and management of this metabolic disorder. **Aims and Objectives:** Our aim was to elucidate the relationship of H_2S synthesizing activity in plasma and fasting blood glucose in type 2 diabetic patients. **Materials and Methods:** Sixty-two newly diagnosed type 2 diabetes patients and equal number of non-diabetic controls were included in the study. Enzymatic activity of synthesizing H_2S in plasma was estimated following methods described earlier with further modification and standardization in our laboratory. All other parameters were estimated by using standardized kits. **Results:** FBG, PPBG, HbA_{1c}, Fasting Insulin, H_2S synthesizing activity in plasma in patients are significantly higher ($p < 0.05$) than the corresponding values in healthy controls H_2S synthesizing activity in plasma is positively correlated with fasting plasma glucose and the correlations is significant ($p = 0.05$). **Conclusion:** Our study though a pilot study with a small sample size, has elucidated that the values of H_2S synthesizing activity in plasma are significantly elevated in type 2 diabetic patients and this may help researchers to develop H_2S modifying agents and enzyme inhibitors which may open up new horizon in the treatment modalities of type 2 diabetes mellitus.

Key words: H_2S synthesizing activity, FBG, Type 2 Diabetes

Access this article online

Website: http://nepjol.info/index.php/AJMS

DOI: 10.3126/ajms.v7i5.14674
E-ISSN: 2091-0576
P-ISSN: 2467-9100

INTRODUCTION

Type 2 diabetes mellitus has turned out as a multi-factorial epidemic disease. In India, the steady migration of people from rural to urban areas, the economic boom, and corresponding change in life-style are all affecting the level of diabetes.[1] The prevalence of type 2 diabetes mellitus in India was 51 million people in 2010.[2]

In contrast to its role as poison, hydrogen sulfide (H_2S) is considered as the third gasotransmitter after nitric oxide (NO) and carbon monoxide (CO).[3-5] Though it was first reported in 1982 that it is produced in mammalian tissues, but only now it has emerged as a mediator of important physiologic functions in humans.[6] Experimental evidences have been published implicating H_2S overproduction as a causative factor in the pathogenesis of β-cell death in diabetes.[7,8]

H_2S is produced *in vivo* from L-cysteine by the action of enzymes, cystathionine beta-synthase (CBS) and cystathionine gamma-lyase (CSE). Both of these enzymes are dependent on pyridoxal-5- phosphate. Recent studies have shown two

Address for correspondence:
Dr. Utpal Kumar Biswas, Professor, Department of Biochemistry, Institute of Post Graduate Medical Education and Research, Kolkata, India.
E-mail: drutpalbiswas2010@gmail.com, **Phone:** +91-9051642109

other H_2S-producing enzymes, 3-mercaptopyruvate sulfur transferase (3MST), along with cysteine aminotransferase (CAT), which produces H_2S in the brain as well as in the vascular endothelium.[9,10] H_2S synthesizing activity in plasma represents the enzyme leakage from the tissues involved in the synthesis of H_2S. We have modified and standardized the methods reported earlier. Though several etiological factors of type 2 diabetes mellitus has been well explained, yet a number of recent literatures suggested a potential role of H_2S and H_2S synthesizing enzymes in the etiology and management of this metabolic disorder.[11]

AIMS AND OBJECTIVES

Our aim was to compare H_2S synthesizing activity in plasma with fasting blood glucose levels and to find out if there is any correlation among them in type 2 diabetic patients.

MATERIALS AND METHODS

62 (Sixty-two) newly diagnosed type 2 diabetes mellitus patients attending O.P.D. of Medicine department at N.R.S. Medical College, Kolkata and 62 non-diabetic control subjects were included in the study after obtaining informed consent with prior approval from institutional ethics committee. 5 ml of 8-10 hours fasting blood samples were collected with heparin and centrifuged at 2500 rpm for 5 minutes. Supernatant plasma were aliquoted and stored in -40°C refrigerator until use and tests were performed within a week. Reagents used were stored at 2-8°C and the chromogen (N, N-dimethyl- p- phenylene diamine sulphate) was freshly prepared before the assay.

Inclusion criteria

Patients of age ranging from 20 to 50 years suffering from type 2 diabetes mellitus diagnosed and confirmed by clinical and biochemical tests were included in the study.

Exclusion criteria

Following patients were excluded:

1. patients having other endocrine disorders,
2. pregnant mothers patients with type-1 diabetes mellitus,
3. polycystic ovarian disease,
4. renal failure,
5. malignant disease and
6. patients receiving plasma H_2S level modifying agents and H_2S releasing agents like sodium sulfide, sodium hydrogen sulfide and their parent drugs like, sildenafil, mesalagine, naproxen, indomethacin, diclofenac etc.
7. **Assay of H2S synthesizing activity in plasma:** For this, initially we followed the estimation of plasma H_2S, by a method modified and standardized in our laboratory and reported earlier.[8,12-14] This spectrophotometric method involves the reaction of sulfide with N, N-dimethyl-p-phenylenediamine sulfate in the presence of the oxidising agent Fe3+ in hydrochloric acid to form methylene blue which is read at 670 nm.

Preparation of buffers and reagents for assay of plasma H_2S synthesizing activity

1. 20 m Molar PBS (pH 7.4): Prepared by mixing 80.4 ml of Solution A (prepared by dissolving 11.47 gm of disodium hydrogen phosphate dissolved in 1000 ml of deionized water) to19.6 ml of Solution B (prepared by dissolving 9.08 gm of potassium dihydrogen phosphate to 1000 ml of deionized water), and pH adjusted to 7.4
2. L-cysteine solution (10 mM/l) prepared with deionized water
3. Pyridoxal -5-phosphate (2 mM/l) prepared with deionized water
4. 1% (w/v) Zinc acetate (MW 219.49)
5. 50% TCA(w/v)
6. 10% Sodium hydroxide-NaOH solution prepared
7. From stock supply of HCl (12N i.e 12M), MW 36.5, 7.2 mM and 1.2 mM solutions were prepared.10 microliters of 12M stock HCl diluted with deionized water and volume made upto 100 ml to get 1.2 mM HCl, and 60 microliters of 12M stock HCl diluted with deionized water and volume made upto 100 ml to get 7.2 mM HCl
8. 20 milimol N,N-dimethyl- p- phenylenediamine sulphate in 7.2 mM HCl
9. 30 milimol of $FeCl_3$ dissolved in 1.2 mM of HCl
10. **Assay Procedure for H2S synthesizing activity in plasma:**[8,15] One hundred microliter of plasma was added to 800 microliter of ice-cold PBS buffer (pH 7.4-8.0) in a glass tube. The mixture 900 microliter was incubated at 37°C for 5 minutes and then cooled on ice for 10 minutes. Next 50 microliter of each of L-cysteine (10 mM/l) and pyridoxal-5-phosphate (2 mM/l) were added to make the final volume of 1 ml. Then 1% zinc acetate 300 microliter added to the glass tube which is then capped or sealed with parafilm. The tubes were then transferred to a shaking water bath and kept incubated there for 90 minutes. Then the cap was removed and 500 microliters of 50% trichloroacetic acid was added and the tubes were again left incubated for 60 minutes, next centrifuged at 2500 for 15 to 20 minutes. The tubes are decapped and 50 microliter of 20 mmol N, N-dimethyl- p- phenylene diamine sulphate and 50 microliter of 30 mmol of $FeCl_3$ were added. Again the resulting solution was kept incubated at room temperature for 20 minutes. Then to all glass tubes 110 microliter of 10% NaOH were added and now the solution appeared turbid and ultimately absorbance was

taken at 670 nm. H_2S synthesizing activity of the plasma expressed as micromol per 100 g of protein.

Standardization of assay of H_2S synthesizing activity in plasma

Initially absorbance values obtained from each sample in the technique of assay of H_2S synthesizing activity, were extrapolated from the standard curve of H_2S (Figure 1) and then 30% of the determined value was considered as the synthesizing activity of H_2S in plasma.[11]

Estimation of other parameters

Fasting blood glucose and post prandial blood glucose measured by GOD-POD method using standardized kits, total protein by Biuret standardized kit, HbA_{1C} by ion-exchange chromatography standardized kit and fasting insulin by standardized ELISA kit.

Statistical methods

Data were expressed as mean ± standard deviation (SD), statistical analysis was done using Microsoft Office Excel-2007 and SPSS Statistics version 2020.

RESULTS

In our study the values of FBG, PPBG, HbA_{1C}, Fasting Insulin, H_2S synthesizing activity in plasma in patients are significantly higher ($p< 0.05$)than the corresponding values in healthy controls (Table 1). Figures 2 and 3 elucidates that values of FBG and H_2S synthesizing activity in plasma are positively correlated and the correlation is statistically significant ($p=0.05$).

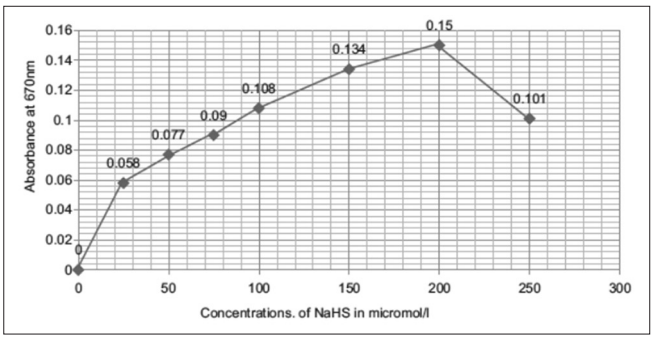

Figure 1: Standard curve of plasma H_2S assay

DISCUSSION

H_2S can be produced in pancreatic β-cells by cystathionine β-synthase (CBS) or cystathionine γ-lyase (CSE). H_2S inhibits insulin release and regulates β-cell survival.[11] Besides inhibiting insulin release H_2S protects β-cells that were chronically exposed to high glucose from apoptotic cell death. Loss of β-cell mass and failures of β-cell function are important in the pathogenesis and/or progression of diabetes mellitus; therefore, molecular analysis of the mechanisms of H_2S production and its protective effects on β-cells may lead to new insights into diabetes mellitus.[11] In addition, the changes in H_2S homeostasis also play a role in the pathogenesis of endothelial injury which developed under the basis of elevated circulation of blood glucose levels in diabetes.[15] Measuring the H_2S synthesizing activity in plasma in streptozotocin treated diabetic rats has been undertaken by researchers in recent times. There are some contradictory findings. One study reported that the streptozotocin induced diabetes in the rat is associated with enhanced tissue hydrogen sulfide biosynthesis.[16] Another study suggested that H_2S synthesis is progressively reduced as diabetic pathology increased.[17]

Recently, a role for H_2S in the etiology of diabetes has been suggested. Pancreatic synthesis of H_2S is markedly elevated in the streptozotocin (STZ) induced diabetic rat,[16] in which biphasic effects on beta cells have been observed; at low concentrations, H_2S inhibited insulin release through K_{ATP} dependent/Ca2+-independent mechanism,[12] whereas higher levels induced beta cell death through endoplasmic- reticular-stress-dependent pathways.[18] In experimental diabetes animal models, both Streptozotocin and Zucker diabetic fatty rats had significantly higher H_2S formation in the pancreas.[16]

Table 1: Clinico-biochemical parameters of patients & controls

Variables	Mean±SD		P value
	Patient (N=62)	Control (N=62)	
Age (years)	44.03±5.27	43.63±5.56	NS
Sex (M/F)	30/32	37/25	
Body mass index (BMI)	24.15±4.09	24.81±2.63	NS
Fasting blood glucose (mg/dl)	134.14±26.88	79.06±12.64	<0.001*
Post prandial blood glucose (mg/dl)	176.08±46.26	108.76±11.16	<0.001*
Glycosylated haemoglobin (%)	6.85±1.14	3.8±0.34	<0.001*
Fasting plasma insulin (micro IU/l)	11.02±7.59	7.67±3.1	0.002*
Plasma H_2S synthesizing activity (micromol/100 g of protein)	11.40±5.38	4.62±1.91	<0.001*
Total protein (g/dl)	6.4±0.44	6.6±0.41	0.008*

Student's t-test was done, * are significant (P<0.05), with 95% confidence level: NS: Not significant

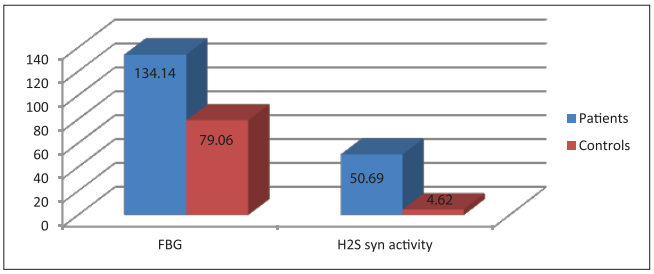

Figure 2: Comparison between patients and controls with respect to fasting blood glucose (FBG) and plasma H_2S synthesizing activity

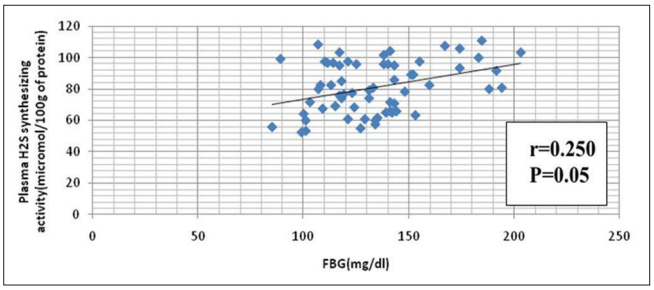

Figure 3: Scatter plot showing correlation between fasting blood glucose and plasma H_2S synthesizing activity

In our study, the H_2S synthesizing activity in plasma of patients is 11.40 ± 5.38 μ mol/100 g of protein which is significantly ($p<0.001$) higher than that in plasma of controls equivalent to 4.62 ± 1.91 μ mol/100 g of protein. The fasting blood glucose levels of the patients (134.14 ± 26.88 mg/dl) are significantly ($P<0.001$) higher than the controls (79.06 ± 12.64 mg/dl) and the values of FBG and H_2S synthesizing activity in plasma are positively correlated and the correlation is significant($p=0.05$).

M. Yusuf et al. has reported that streptozotocin-induced diabetes in rat is associated with enhanced tissue hydrogen sulfide biosynthesis.[16] The activities of H_2S producing enzymes are known to increase under diabetic conditions as reported by Kaneko Y. et al.[19] Elevated hydrogen sulfide levels in plasma were reported in patients with proliferative diabetic retinopathy.[20] In streptozotocin induced type 2 diabetic experimental rats in the plasma it has been earlier reported from our laboratory that along with reduced plasma H_2S levels, there is reduction in the H_2S synthesizing activity.[8] Some other studies have reported reduced H_2S levels in the plasma as well as in the tissues in this condition like the one reported by *S*ushil K. Jain which demonstrated lower circulatory levels of H_2S in type 2 diabetic patients,[21] but associated levels of pro-inflammatory cytokines may have affected the outcome. But elsewhere it has been reported that biosynthesis of H_2S at the tissue level in diabetic animal model has shown both increase of H_2S concentrations and increment in the activity and expression of the enzymes related with its synthesis.

CONCLUSION

Though our study consisted of less sample size, it was able to demonstrate the link between H_2S and diabetes, furthermore elucidating significantly increased synthesis of H_2S in type 2 diabetic patients. This is an eye-opener which might pave way for researchers to develop H_2S modifying agents and enzyme inhibitors opening up new horizon in the treatment modalities of type 2 diabetes mellitus.

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Authors Contribution:
UKB - Concept and design of the study, statistical analysis, helped manuscript preparation and critical revision of the manuscript; **PS, SN, PG -** Concept, collected data, literature search, statistically analyzed and interpreted, prepared first draft of manuscript; **SDG & SS -** Helped in standardization and statistical analysis and helped in preparing first draft of manuscript. **TJS -** Conceptualized study, helped in patient selection; **AK -** Edited the manuscript, critical revision of the manuscript, helped in final draft of manuscript.

Source of Support: Nil. **Conflict of Interest:** None.