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Clinical Implication of Plasma Hydrogen Sulfide Levels in Japanese Patients with Type 2 Diabetes

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Abstract

Objective The goal of the present study was to investigate the plasma hydrogen sulfide (H_2S) levels in patients with type 2 diabetes, as the plasma H_2S levels in Japanese patients with type 2 diabetes remain unclear.

Methods The plasma H_2S levels were measured in 154 outpatients with type 2 diabetes and 66 outpatients without diabetes. All blood samples were collected in the outpatient department from 09:00 to 10:00. The patients had fasted from 21:00 the previous evening and had not consumed alcohol or caffeine or smoked until sample collection. The plasma H_2S levels were measured using the methylene blue assay. The plasma H_2S levels were determined in triplicate, and the average concentrations were calculated against a calibration curve of sodium sulfide.

Results The patients with type 2 diabetes showed a progressive reduction in the plasma H_2S levels (45.1±15.5 μM versus 54.0±26.4 μM, $p<0.05$), which paralleled poor glycemic control. There was a significant correlation between a reduction in the plasma H_2S levels and the HbA1c levels ($\beta=-0.505$, $p<0.01$), Furthermore, a reduction in the plasma H_2S levels was found to be related to a history of cardiovascular diseases in patients with type 2 diabetes (39.9±13.8 μM versus 47.5±15.9 μM, $p<0.01$).

Conclusion Collectively, the plasma H_2S levels were reduced in patients with type 2 diabetes, which may have implications in the pathophysiology of cardiovascular disease in diabetic patients. The trial was registered with the University Hospital Medical Information Network (UMIN no. #000020549).

Key words: hydrogen sulfide, type 2 diabetes, cardiovascular disease

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Introduction

Hydrogen sulfide (H_2S) is a colorless, watersoluble gas with a smell of rotten eggs and is an endogenously produced labile diffusible mediator with multiple roles in the cardiovascular system in health and disease. H_2S is endogenously generated, and cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST) are key enzymes involved in its biological production (1-3). Although the physiological effect of H_2S is not yet clear, it is known to influence cardiovascular homoeostasis, such as through vasodilation, pro-apoptotic activities in vascular smooth muscle, leukocyte-endothelium interaction, and suppression of oxidative stress (3-6). We previously reported that, in hyperglycemic endothelial cells and streptozotocin-induced diabetes in rats, hyperglycemia is associated with increased H_2S degradation, as the H_2S concentrations in the media and plasma were decreased. In addition, H_2S replacement relieved oxidative stress and improved the endothelial metabolic states. Brancaleone et al. demonstrated that vascular dilation, H_2S biosynthesis, and H_2S endogenous production progressively contribute to the severity of diabetes in non-obese diabetic mice (7). However, little has been reported on plasma H_2S levels in type 2 diabetic patients, particularly Japanese patients.

In the present study, we examined the plasma H_2S levels in patients with type 2 diabetes and evaluated the relevance of H_2S to the history of cardiovascular disease in these patients.

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Table 1. Clinical Characteristic of the Study Participants.

	Type 2 diabetes	Non-Type 2 diabetes	p value
Patients (M/F)	154 (67/87)	66 (25/41)	< 0.001
Age (years)	61.7 ± 13.6	52.5 ± 14.8	< 0.001
Body mass index (kg/m^2)	25.3 ± 4.7	24.1 ± 5.6	< 0.05
HbA1c, % (NGSP)	7.6 ± 1.6	5.7 ± 0.8	< 0.001
Duration of diabetes (years)	13.2 ± 9.1	-	-
eGFR (mL/min/1.73m^2)	78.3 ± 29.3	84.9 ± 32.6	0.136
Urinary albumin excretion (μg/g･Cre)	28.5 (3.0-151.8)	4.0 (3.0-8.0)	< 0.001
ACEI or ARB, n (%)	85 (55.2%)	13 (19.6%)	< 0.001
Statins, n (%)	65 (42.2%)	22 (33.3%)	< 0.01
LDL-C (mg/dL)	102.3 ± 28.9	114.3 ± 45.0	0.180
HDL-C (mg/dL)	55.9 ± 14.6	69.5 ± 23.8	< 0.001
Triglyceride (mg/dL)	130.4 ± 67.4	127.1 ± 76.5	0.239
Systolic Blood Pressure (mmHg)	131.5 ± 15.5	119.8 ± 20.4	< 0.001
Diastolic Blood Pressure (mmHg)	73.5 ± 11.1	67.8 ± 13.0	< 0.05

Data are mean ± SD or median and interquartile range.

HbA1c: hemoglobin A1c, eGFR: estimated glomerular filtration rate,

ACEI: angiotensin converting enzyme inhibitor, ARB: angiotensin II receptor blocker,

LDL-C: low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol

Materials and Methods

This randomized pilot study was conducted on 200 randomly selected patients with type 2 diabetes from the 626 outpatients who had visited this institution for type 2 diabetes and 100 without diabetes from the 308 outpatients without diabetes who had visited for hypertension, dyslipidemia, or thyroid disease controlled satisfactorily by medication. The exclusion criteria were 1) severe ketosis and diabetic coma, 2) severe complication of diabetes, 3) renal insufficiency (serum creatinine level >1.5 mg/dL in men or >1.3 mg/dL in women) and liver dysfunction, 4) severe infection, 5) pregnant or nursing women and those who might be pregnant, 6) alcoholism, and 7) any patients whom the investigators judged to be inappropriate for this study. Ultimately, 154 patients with type 2 diabetes and 66 without diabetes were enrolled.

All of the blood and samples were collected in the outpatient department from 09:00 to 10:00. The patients had fasted from 21:00 the previous evening and had not consumed alcohol or caffeine or smoked until sample collection. This study was approved by the Ethics Committee of Dokkyo Medical University (Tochigi, Japan). The Clinical Trial registration no. is UMIN000020549. All of the subjects provided their written informed consent. This study was designed in accordance with the principles stated in the Declaration of Helsinki.

The blood samples to measure the plasma H_2S levels were collected in an EDTA anti-coagulated tube. The plasma H_2S levels were determined in triplicate, and the average concentrations were calculated against a calibration curve of sodium sulfide (Sigma-Aldrich, MO, USA).

Briefly, as described previously (7-10), the plasma samples (100 μM) were added to sealed Eppendorf tubes containing 200 μL zinc acetate (1% wt/vol). Then, 100 μL N,N-dimethyl-*p*-phenylenediamine sulfate (20 mM, in 7.2 M HCl) and 133 μL FeCl3 (30 mM, in 1.2 M HCl) were added. After incubation at 37℃ in the dark for 30 minutes, the samples were centrifuged at 5,000 g for 10 minutes, and the absorbance was measured at 670 nm.

The data are presented as the means ± standard deviation (SD). The differences between the groups were analyzed using Student's paired or unpaired *t*-test. The differences in the non-parametric data were analyzed using the Mann-Whitney U-test and Wilcoxon's matched pairs test. Univariate and multivariate logistic regression analyses were performed to assess whether each clinical marker correlated with the plasma H_2S levels. Values of $p<0.05$ were considered significant. All of the analyses were performed using Prism 6 (GraphPad Software, Inc., San Diego, CA, USA) and Stat Mate V (Nihon 3B Scientific, Inc., Niigata, Japan).

Results

The participants' demographics are shown in Table 1. The plasma H_2S levels were significantly lower in patients with type 2 diabetes than in those without (45.1±15.5 μM versus 54.0±26.4 μM, $p<0.05$; Figure a). All of the patients with type 2 diabetes having poor glycemic control exhibited a progressive reduction in plasma H_2S levels as well as HbA1c levels (Figure b). Furthermore, the plasma H_2S levels in patients with type 2 diabetes with a history of cardiovascular disease (non-fetal cerebrovascular disease, coronary artery disease, and peripheral artery disease) were significantly lower than those in patients with type 2 diabetes without cardiovascular disease (39.9±13.8 μM versus 47.5±15.9 μM, $p<0.01$) (Figure c). In the univariate analysis, the plasma H_2S levels were negatively correlated with the HbA1c level (γ=-0.730, $p<0.001$), duration of diabetes (γ=-0.689, $p<0.001$), systolic blood pressure (γ=-0.203, $p<0.05$), and diastolic blood pressure (γ=-0.165, $p<0.05$). A stepwise regression analysis including significant variables was performed. The HbA1c level (β=0.505, $p<0.01$) was an independent de-

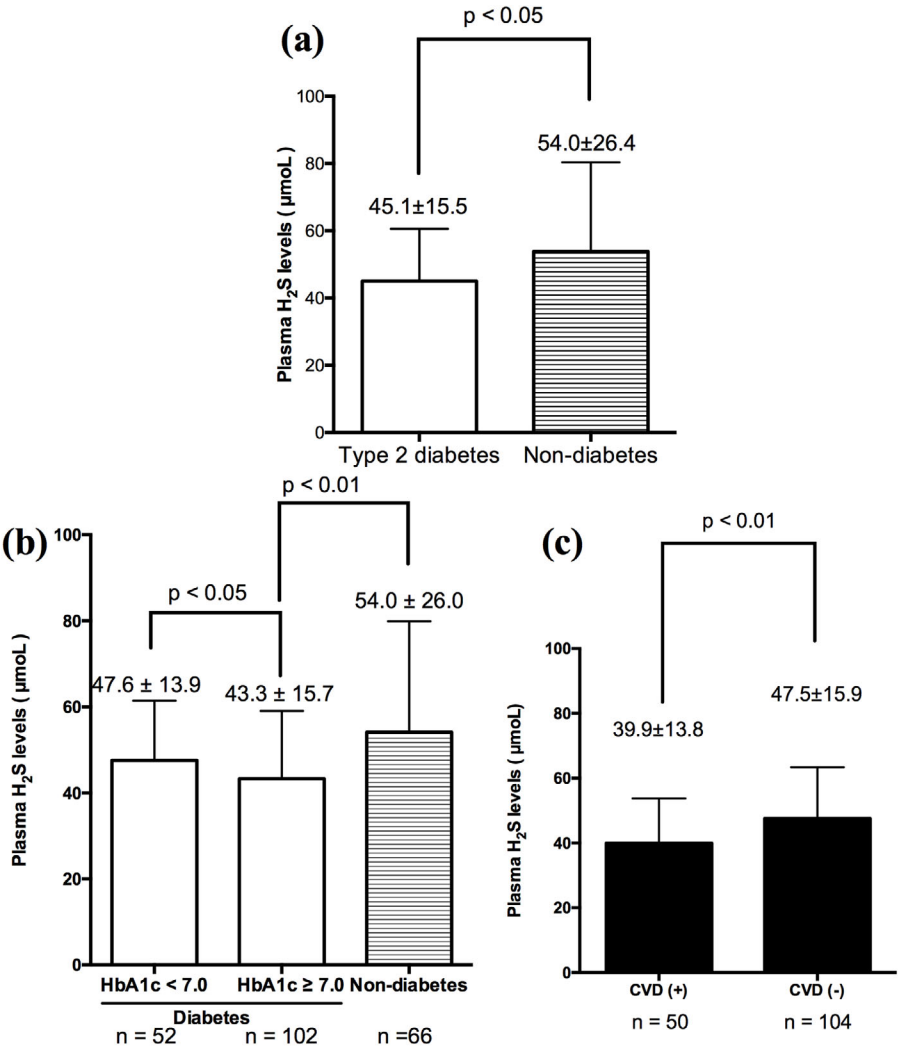

Figure. **The plasma H_2S levels in patients with and without type 2 diabetes. (a) A comparison of the patients with and without type 2 diabetes. (b) The influence of glycemic control on the plasma H_2S levels. (c) The relationship between a history of cardiovascular disease (CVD) and the plasma H_2S levels in patients with type 2 diabetes.**

terminant of plasma H_2S levels (Table 2).

Discussion

H_2S is the most recent of three endogenously produced molecules to be discovered and termed a gasotransmitter, along with nitric oxide (NO) and carbon monoxide (CO) (11). While initially considered to be toxic, researchers have discovered that these gasotransmitters are endogenously produced at low levels and induce compensatory and protective physiological changes (12). The first gasotransmitter, NO, was discovered in the 18th century but was found in 1987 to be an endogenous vasodilator in mammals through its generation by NO synthase (13). NO is not only a mediator of endothelium-dependent vasodilation but also has anti-inflammatory and antithrombotic effects, as well as an impact on the development of atherosclerosis (14). The second gasotransmitter, CO, is a vital cardiovascular mediator that upregulates the protective mechanisms at a low physiologic concentration (15). The third gasotransmitter, H_2S, was found to play an important role in mediating physiological processes. H_2S has been shown to modulate the metabolic state, vascular reactivity, and systolic blood pressure (1, 16). The present data demonstrated that plasma H_2S levels are significantly reduced in patients with type 2 diabetes, particularly in those with a history of cardiovascular disease. This study is the first report to determine the plasma H_2S levels in a large group of Japanese patients with type 2 diabetes.

H_2S is an important endogenous vasodilatory intermediate that induces endothelial vasodilatation though the K_{ATP} channel *in vitro* and *in vivo* (17). Type 2 diabetes is likely to be associated with the development of hypertension, hypercholesterolemia, diabetes-related complications, and heart failure (18, 19). However, the precise molecular mechanism underlying why these complications occur in patients with diabetes is still unclear. The current study revealed that plasma H_2S levels are reduced in patients with type 2 diabetes mel-

Table 2. Univariate and Multivariate Analysis for Association with Each Variable with Hydrogen Sulfide Levels.

	Univariate		Multivariate	
Variable	r	p value	β	p value
Age (years)	-0.069	0.387	-	-
Body mass index (kg/m^2)	-0.067	0.401	-	-
HbA1c, % (NGSP)	-0.730	< 0.001	-0.505	0.001
Fasting glucose per mg/dL	0.050	0.438	-	-
duration of diabetes yrs	-0.689	< 0.001	0.006	0.937
Systolic Blood Pressure (mmHg)	-0.203	< 0.05	-0.108	0.154
Diastolic Blood Pressure (mmHg)	-0.165	< 0.05	-0.090	0.311
LDL-C (mg/dL)	-0.049	0.552	-	-
HDL-C (mg/dL)	-0.045	0.59	-	-
Triglyceride (mg/dL)	0.128	0.08	-	-
eGFR (mL/min/1.73m^2)	-0.003	0.975	-	-
Urinary albumin excretion (μg/g・Cre)	-0.138	0.102		
R^2			0.58	

HbA1c: hemoglobin A1c, eGFR: estimated glomerular filtration rate,
LDL-C: low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol

litus compared to those without, which may influence the occurrence of atherosclerotic disease. Many studies have reported the beneficial effects of H_2S replacement therapy using H_2S donors in several organs and have also observed neuroprotective effects (20) of the liver and lungs from ischemia-reperfusion injury in a rat model (21, 22). Furthermore, H_2S has been shown to be an antioxidant itself and can upregulate the antioxidant protective mechanism (23). These effects of H_2S may be salutary to a number of cardiovascular disease states, including diabetes.

Our current study showed that plasma H_2S levels negatively correlated with HbA1c, duration of diabetes, and systolic and diastolic blood pressures. The most interesting finding was that glycemic control was the major determinant of the plasma H_2S levels, which reflect the HbA1c levels. In addition, Brancaleone et al. reported that biosynthesis of H_2S is impaired in diabetic mice, and endogenous H_2S production is more impaired in diabetic mice with more severe glycemia than in those with less severe glycemia (6). In our study, the peripheral endothelial function was assessed in 32 of 154 type 2 diabetes patients using reactive hyperemia peripheral arterial tonometry (RH-PAT) with an EndoPAT2000 (Itamar Medical, Caesarea, Israel) as described previously (24). The endothelial function was also positively correlated with the plasma H_2S levels (γ=0.467, p<0.01). A previous study demonstrated that the plasma H_2S levels likely contribute to the endothelium-dependent relaxant function in diabetic rodents (6, 25), which is consistent with our findings for type 2 diabetes patients.

Regarding the possible clinical application of H_2S-releasing drugs, H_2S exerts a number of cytoprotective and anti-inflammatory effects in many organ systems. A number of H_2S-releasing derivatives of existing drugs have been developed and extensively tested in preclinical models (26). H_2S-releasing drugs are expected to have the beneficial effects of endogenous gaseous mediators in the gastrointestinal tract, which include reducing the gastrointestinal ulceration and bleeding caused by nonsteroidal anti-inflammatory drugs, as well as in the cardiovascular-protecting systems (27).

Several limitations associated with the present study warrant mention. First, the plasma H_2S levels in the present study were measured using the methylene blue assay, which indicates the sulfide concentrations through colorimetry and may not be entirely accurate because of the interference and artifacts (28). Fluorescent probe detection is another method that could be used for H_2S level detection. However, H_2S catabolism occurs rapidly, which could result in fluctuation of the H_2S concentration; therefore, it may be difficult to accurately analyze this important molecule (29). Second, we were unable to assess the endothelial function in all of the type 2 diabetes patients using the EndoPAT2000.

In conclusion, this study showed that the plasma H_2S levels are significantly reduced in type 2 diabetes patients, particularly in those with a history of cardiovascular disease. The plasma H_2S levels in patients with type 2 diabetes may reflect the HbA1c levels. Further studies are warranted to investigate the relationship between the plasma H_2S levels and diabetes and related complications, particularly cardiovascular diseases.

The authors state that they have no Conflict of Interest (COI).

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