Original Article

Correlation of Lower Concentrations of Hydrogen Sulfide with Activation of Protein Kinase CβII in Uremic Accelerated Atherosclerosis Patients

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Abstract

Background: Hydrogen sulfide (H_2S) plays a protective role in chronic hemodialysis (CHD) patients. In this study, we further investigate the relationship between H_2S and conventional protein kinase CβII (cPKCβII) in CHD patients with uremic accelerated atherosclerosis (UAAS).
Methods: A total of 30 healthy people, 30 CHD patients without AS and 30 CHD patients with AS (CHD + AS) were studied. Plasma H_2S was measured with a sulfide sensitive electrode, and cPKCβII membrane translocation was detected by Western blotting.
Results: Plasma H_2S in CHD + AS group was significantly lower than that in CHD patients. cPKCβII membrane translocation in CHD + AS group increased significantly compared with CHD group. Plasma H_2S concentration was negatively correlated with cPKCβII membrane translocation in CHD + AS patients.
Conclusions: These findings suggest a possible linkage between H_2S metabolism and cPKCβII activation, which may contribute to the development of UAAS in CHD patients.

Key words: Hemodialysis; Hydrogen Sulfide; Protein Kinase CβII; Uremic Accelerated Atherosclerosis

Introduction

It is well-known that cardiovascular diseases are the leading cause of death in chronic hemodialysis (CHD) patients and accelerated atherosclerosis (AS) is the major contributing factor for mortality in these dialysis patients.[1] The mortality caused by cardiovascular disease in the death of end-stage renal disease (ESRD) patients accounted for about 50%.[2]

Hydrogen sulfide (H_2S) is considered as the third endogenous gaseous transmitter besides nitric oxide (NO) and carbon monoxide,[3] which exerts a wide range of physiological functions *in vivo*, such as relaxing vascular smooth muscle, inhibiting proliferation of vascular smooth muscle cells, and lowering blood pressure (BP).[4] It has been reported that the decrease of H_2S in the plasma of hemodialysis patients may have relevance to the pathogenesis of the uremic syndrome manifestations, such as hypertension and AS.[5] We also have previously reported H_2S metabolism abnormalities may contribute to the development of uremic accelerated AS (UAAS) in CHD patients with diabetic nephropathy.[6]

Protein kinase C (PKC) is a family of serine/threonine kinase comprised of 10 isoforms, they differ in requirement of Ca^{2+} and phospholipids for activation, and may partake of protective or deleterious effects in an isoform-specific manner.[7] Of the various PKC isoforms, conventional protein kinase CβII (cPKCβII) has been shown to contribute to the pathology associated with heart failure,[8] and its inhibition may benefit patients with heart failure.[9] Study from Harja *et al*. further demonstrated that activation of cPKCβII in the pathogenesis of AS, and blockade of cPKCβII may be beneficial in AS.[10] However, the function of cPKCβII in UAAS remains to be determined. Accordingly, the role of cPKCβII activation in UAAS was investigated, and the correlation of H_2S and cPKCβII activation was elucidated in this study.

Methods

Data sources

A total of 30 CHD patients without AS and 30 CHD patients

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with AS (CHD + AS) were enrolled in the study if they were more than 18 years of age, had no residual renal function, and had maintained hemodialysis for more than 3 months with ESRD were diagnosed as CHD. CHD patients with AS were defined as localized thickening of intima-media thickness (IMT) ≥1.2 mm that did not uniformly involve the whole wall of the carotid artery.

Patients were not included in the study if they had heart failure, a recent acute coronary event, cancer, autoimmune disease, and active infection. A standard questionnaire was used for each participant to obtain systematic information regarding conventional cardiovascular risk factors, including hyperlipidemia, hypertension, diabetes, and family history of cardiovascular disease.

As a normal control group, age- and gender-matched, 30 healthy individuals were enrolled in this study.

The study was approved by the ethics committee of Beijing Chao-Yang Hospital, Capital Medical University, and written informed consent was obtained from each participant.

Hydrogen sulfide concentration measurement

The blood of patients was drawn prior to the mid-week dialysis session. Once blood was drawn in plastic vacutainers using EDTA (1 mg/ml of blood), plasma was immediately obtained through brief 5 min centrifugation at 500 × *g* and rapidly added to the assay mixture. Plasma H_2S concentration was measured with a sulfide sensitive electrode as described by Li *et al*.[11] with modifications. Briefly, 0.5 ml of plasma was added into a test tube containing 0.5 ml of 0.04 g NaOH, 0.035 g EDTA and 0.05 g ascorbic acid. The sulfide sensitive electrode and a reference electrode immersed into the sample together, and record the serum H_2S concentration until the reading is stable. H_2S concentration was calculated against a calibration curve obtained with known H_2S concentrations in a range between 5 and 100 μmol/L, utilizing the H_2S donor NaHS.[12,13] Standard curves were repeated daily with triplicate measurement for each point, and freshly made solutions were utilized at all times.

Sample preparation and Western blotting analysis

Peripheral blood mononuclear cells (PBMCs) were separated from blood samples by lymphocyte separation medium, which were used to detect the cPKCβII activation *in vitro*. Cells were washed twice with ice-cold PBS and solubilized in buffer A (5 mmol/L Tris-Cl, pH 7.5, containing 2 mmol/L dithiothreitol, 2 mmol/L EDTA, 1 mmol/L EGTA, 5 g/ml each of leupeptin, aprotinin, pepstatin A and chymostatin, 50 mmol/L potassium fluoride, 50 mmol/L okadaic acid, 5 mmol/L sodium pyrophosphate). Homogenates were centrifuged at 30,000 × *g* for 30 min at 4°C. The supernatants were collected as the cytosolic fraction. The pellets were re-suspended in buffer B (Buffer A containing 0.5% Nonidet P-40 [Sigma-Aldrich Corp., St. Louis, MO, USA]) before being sonicated and centrifuged at 30,000 × *g* for 30 min at 4°C again. The resulting supernatants were obtained as the particulate fraction. Protein concentration was determined by BCA kit (Pierce Company, Rockford, IL, USA) with albumin diluted in lysis buffer as standard. Proteins (40 μg) from each sample per lane were loaded on 10% SDS-polyacrylamide gel electrophoresis. The gels were electrophoresed, and then transferred onto polyvinylidene difluoride membrane (GE Healthcare) at 4°C. After rinses with TTBS (20 mmol/L Tris-Cl, pH 7.5, 0.15 mol/L NaCl and 0.05% Tween-20), the transferred polyvinylidene difluoride membrane was blocked with 10% nonfat milk in TTBS for 1 h and incubated with the corresponding primary antibodies for 4 h. The horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Stressgen Biotechnologies Corporation, Victoria, BC, Canada) was used as second antibodies. Following incubation with the primary and secondary antibodies, the enhanced chemiluminescence kit (GE Healthcare, British) was employed to detect the signals. To verify equal loading of protein, the blots were reprobed with primary monoclonal antibody against β-actin (Sigma-Aldrich Company, USA).

Statistical analysis

All the data were analyzed using a statistical software package (SPSS for Window, Version 13.0, spss Inc., Chicago, IL, USA). For membrane translocation, the ratio of cPKCβII (band density in particulate/bands densities in both particulate and cytosol) in the Control group was expressed and normalized as 100%. The data from other group were expressed as a percentage of that from the control group. For protein expression level, the protein ratio (band density of protein/band density of β-actin) was also expressed as 100% in the control group. Measurement data were presented as mean ± standard deviation (SD). Comparisons were performed using one-way analysis of variance (ANOVA) with *post-hoc* analysis (LSD) and independent-samples *t*-test. In addition, bivariate correlation analysis was performed. A $P < 0.05$ was regarded as statistically significant.

Results

Subject characteristics

A total number of 60 patients (30 CHD, 30 CHD + AS) with a mean age of 47.2 ± 12.1 years (range 20–71 years) and a mean dialysis period of 42.7 ± 17.8 months (range 5–84 months) were included in this study. Control group consisted of 10 men and 10 women. CHD group consisted of 18 men and 12 women; the mean age was 47.3 ± 11.9 years and average dialysis period was 40.3 ± 18.0 months. CHD + AS group consisted of 19 men and 11 women; the mean age was 47.2 ± 12.5 years and average dialysis period was 45.0 ± 17.7 months. There was no significant difference between CHD and CHD + AS group in terms of age, sex ratio, dialysis duration, smoking, body mass index , Kt/V, Hb, serum creatinine, blood urea nitrogen, triglyceride (TG), total cholesterol (TC), etc., [Table 1]. Patients were not included in the study if they had heart failure, a recent acute coronary event, cancer, autoimmune disease, and active infection. A standard questionnaire was used for every participant to obtain systematic information regarding conventional cardiovascular risk factors, including hyperlipidemia, hypertension, diabetes, and family history of cardiovascular disease.

Table 1: Characteristics of both study groups

Items	CHD group (n = 30)	CHD + AS group (n = 30)	t/χ^2 value	P
Age (years)	47.3 ± 11.9	47.2 ± 12.5	0.021	0.983
Gender (male/female)	18/12	19/11	0.071	0.791
Dialysis duration (months)	40.3 ± 18.0	45.0 ± 17.7	1.021	0.311
BMI (kg/m^2)	23.5 ± 2.3	23.1 ± 1.4	0.888	0.378
Smoking, n (%)	6 (0.2)	7 (23.3)	0.098	0.754
Hypertension, n (%)	16 (53.3)	12 (40.0)	1.071	0.301
SBP (mmHg)	140.6 ± 7.6	142.9 ± 11.1	0.911	0.366
DBP (mmHg)	80.5 ± 7.4	82.9 ± 5.8	1.406	0.165
Kt/V	2.3 ± 0.3	2.4 ± 0.3	0.769	0.445
Hemoglobin (g/L)	115.7 ± 8.1	119.3 ± 9.0	1.630	0.108
Albumin (g/L)	33.9 ± 2.2	34.9 ± 3.5	1.289	0.202
Creatinine (μmol/L)	885.1 ± 103.7	905.3 ± 101.8	0.763	0.449
BUN (mmol/L)	24.3 ± 5.7	23.8 ± 4.4	0.385	0.702
TG (mmol/L)	1.44 ± 0.61	1.30 ± 0.71	0.792	0.431
TC (mmol/L)	3.90 ± 1.02	3.92 ± 0.81	0.083	0.934
LDL-C (mmol/L)	2.11 ± 0.49	2.21 ± 0.57	0.767	0.446
RASI, n (%)	26 (86.7)	26 (76.7)	1.002	0.317
CCB, n (%)	28 (93.3)	25 (83.3)	1.456	0.228
β-blocker, n (%)	4 (13.3)	8 (23.3)	1.002	0.317

CHD: Chronic hemodialysis; BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; BUN: Blood urea nitrogen; TG: Triglyceride; TC: Total cholesterol; LDL-C: Low-density lipoprotein cholesterol; CCB: Calcium channel blocker; RASI: Renin abguitensin system inhibitor; AS: Atherosclerosis.

As a normal control group, age- and gender-matched, 30 healthy individuals (15 females and 15 males) were enrolled in this study.

Hydrogen sulfide concentration in chronic hemodialysis and chronic hemodialysis + atherosclerosis patients

As shown in Figure 1, plasma H_2S level in CHD patients was significantly lower than the control group ($P < 0.05$). Meanwhile, the plasma H_2S level in CHD + AS group was significantly lower than that in CHD group ($P < 0.05$).

Conventional protein kinase CβII activation in chronic hemodialysis and chronic hemodialysis + atherosclerosis patients

Compared with the control group, the membrane translocation (activation) of cPKCβII in CHD group showed an increase, and the increase of cPKCβII membrane translocation in CHD + AS group more obvious (Figure 2, $P < 0.05$).

Correlations between hydrogen sulfide concentration and conventional protein kinase CβII activation in chronic hemodialysis + atherosclerosis patients

In CHD + AS patients, the bivariate correlation analysis showed that cPKCβII activation was negatively correlated with plasma H_2S ($r = -0.970$, $P = 0.000$). No correlation with age, gender, dialysis duration, serum TG, TC, smoking, and hypertension [Table 2].

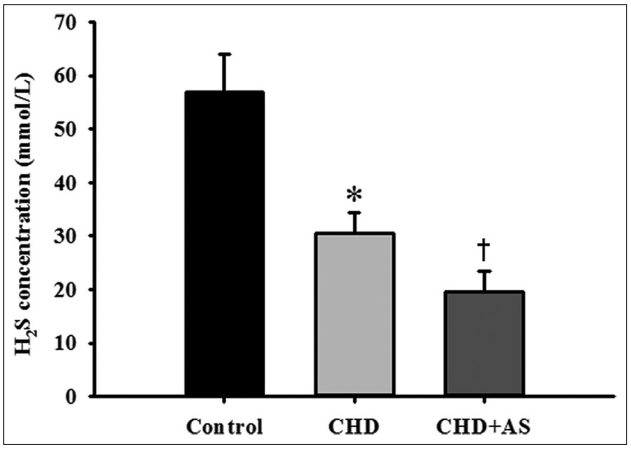

Figure 1: The hydrogen sulfide (H_2S) concentration in control, chronic hemodialysis (CHD) and CHD + atherosclerosis (AS) group. The plasma H_2S contents of the control, CHD and CHD + AS group were measured with a sulfide sensitive electrode (*$P < 0.05$ vs. control group; †$P < 0.05$ vs. CHD group).

Discussion

Chronic kidney disease (CKD) is associated with accelerated cardiovascular risk. The prevalence of cardiovascular disease is 10–20 times greater in patients with CKD compared with people with normal kidney function.[14] Data from prospective studies demonstrated that cardiovascular diseases remain the most common cause of morbidity and mortality in patients with ESRD receiving dialysis, accounting for 40%.[15] AS is associated with the increase of the IMT, and eventually leading to luminal obstruction with consequent ischemic events, such as myocardial infarction and stroke. Lindner *et al*. confirmed that AS was the main cause of cardiovascular disease in patients with CKD, and its progression was accelerated by long-term dialysis.[16] Subsequent investigations elucidated abnormal atherosclerotic pathology in patients with CKD may be classified as AS, arteriosclerosis, and vascular calcification[17-20] Recent evidence further suggested that there is an increased incidence and accelerated progress of AS in patients with ESRD receiving dialysis compared with that of the conventional atherosclerotic cardiovascular disease.[21]

Hydrogen sulfide is an endogenous gas with modulating actions,[22] which has been proposed as an antioxidant due to its ability to protect against oxidative stress and to react with oxidized thiols forming hydrodisulfide.[23] H_2S is synthesized from L-cysteine by two pyridoxal-5'-phosphate-dependent enzymes, cystathionine γ-lyase (CSE) or cystathionine β-synthase (CBS).[24] CBS activity is predominant in H_2S synthesis in the central nervous system whereas CSE is the major H_2S synthesis enzyme in the cardiovascular system.[25] A variety of studies have shown the physiological and pathophysiologic functions, including regulation of BP,[26] renal damage,[27] and neurodegenerative diseases.[28,29] H_2S can decrease the cardiovascular risk through protecting the L-NAME-induced hypertensive rats against liver injury via

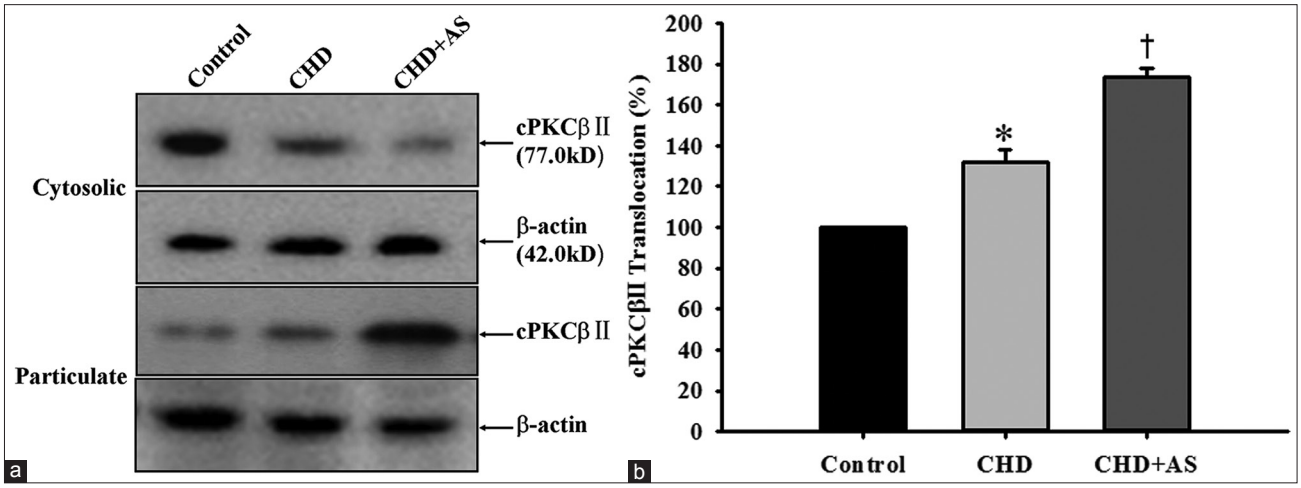

Figure 2: The membrane translocation of conventional protein kinase CβII (cPKCβII) in control, chronic hemodialysis (CHD) and CHD + atherosclerosis (AS) group. (a) The protein contents in cytosolic and particulate fraction of PBMCs were tested by Western blotting; (b) Quantitative analysis showed that cPKCβII membrane translocation in CHD + AS group increased significantly compared with CHD group ($^*P < 0.05$ vs. Control group; $^\dagger P < 0.05$ vs. CHD group).

Table 2: Correlation coefficients for cPKCβII and other variables in CHD + AS patients

Variables	*r*	*P*
H_2S	−0.970	0.000
Age	−0.334	0.072
Dialysis durations	0.074	0.697
SBP	−0.171	0.367
DBP	0.263	0.136
TG	0.136	0.475
TC	0.106	0.576
LDL-C	0.162	0.394

SBP: Systolic blood pressure; DBP: Diastolic blood pressure; TG: Triglyceride; TC: Total cholesterol; LDL-C: Low-density lipoprotein cholesterol; H_2S: Hydrogen sulfide; cPKCβII: Conventional protein kinase CβII; CHD: Chronic hemodialysis; AS: Atherosclerosis.

NO/endothelial NO synthase pathway.[30] The deficiency of H_2S was involved in the pathogenesis of AS,[31,32] and the CSE/H_2S pathway participates in the development and progression of AS in apolipoprotein E knock-out mice.[33] It's worth noting that the low blood level of H_2S was observed in hemodialysis patients,[5] and this declining trend may correlate to the prevalence of hypertension and AS, which are important factors influencing the high cardiovascular mortality present in CKD patients. Meanwhile, in accordance with our previous study,[6] we also found that the decrease of plasma H_2S in CHD patients and this decrease was more significant in CKD patients with AS, which prompted that decrease of H_2S might be an important cardiovascular risk factor in CHD patients with hemodialysis.

Protein kinase C is a family of serine/threonine kinase comprised of 10 isoforms, they differ in requirement of Ca^{2+} and phospholipids for activation, and has a key role in many cellular functions via signal transduction pathways.[34] cPKCβII belongs to the conventional subgroup of the PKC family, and is an important component of the signal transduction pathways response to hypoxic or ischemic stimulation and contribute significantly to the pathogenesis of stroke, cardiovascular disease[35] and diabetic nephropathy.[36] Of note, the deficiency of cPKCβII in mice results in a significant reduction in the progression of AS.[10] Moreover, there is an increasing interest in developing cPKCβII inhibitor for the therapy of AS-associated diseases including diabetes and cardiovascular diseases, and challenges will be posed to raise prospects for future therapeutics. Pigs treated orally with a cPKCβII inhibitor RBX have a significantly better recovery of myocardial contractility and myocardial performance 3 months after infarction injury compared to vehicle-treated pigs.[37] In obesity or hyperlipidemia-induced AS mice, the cPKCβII inhibitor in combination with SOC, can help reduce fat accumulation, improve glucose tolerance, decrease hepatosteatosis and suppress foam cell formation.[38] Meanwhile, cPKCβII inhibitor was helpful to reduce damage secondary to endothelial dysfunction or VSMCs proliferation in patients with AS due to long-term smoking, hypertension or diabetes.[37,39] Therefore, cPKCβII specific inhibitors have been clinically investigated on AS-associated diseases.[40] Nevertheless, treatment with the cPKCβII inhibitor, ruboxistaurin, did not significantly change endothelium-dependent or endothelium-independent vasodilation or blood-based markers of inflammation, fibrinolysis, endothelial damage, and oxidative stress in either diabetic or healthy subjects.[41] It is supposed that the therapeutic effects of cPKCβII inhibition in diabetic patients may be mediated through different and endothelial cell-independent mechanisms. Based on these studies above, and because that the hallmark of PKCs activation is its reversible translocation to the plasma membrane, we detected the cPKCβII membrane translocation of PBMCs in CHD patients with or without AS in this study. We found the activation of cPKCβII was also involved in the process of AS in CHD patients with hemodialysis.

A study from Pan *et al*. prompted that H_2S preconditioning can activate PKCs in cardiomyocytes via different

signaling mechanisms, and protect the heart against ischemia-reperfusion insults partly by ameliorating intracellular Ca^{2+} handling.[42] Similarly, in our present study, we found that the cPKCβII activation was negatively correlated with plasma H_2S in CHD + AS patients.

In summary, these findings in this study suggest a possible linkage between H_2S metabolism and cPKCβII activation, which may contribute to the development of UAAS in CHD patients.

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