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

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Background: Hydrogen sulfide (H2S) plays a protective role in chronic hemodialysis (CHD) patients. In this study, we further investigate the relationship between H2S 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 H2S was measured with a sulfide sensitive electrode, and cPKCβII membrane translocation was detected by Western blotting.

Results: Plasma H2S 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 H2S concentration was negatively correlated with cPKCβII membrane translocation in CHD + AS patients.

Conclusions: These findings suggest a possible linkage between H2S 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 (H2S) 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 H2S in the plasma of hemodialysis patients may have relevance to the pathogenesis of the uremic syndrome manifestations, such as hypertension and AS.[3] We also have previously reported H2S 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 Ca2+ 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 H2S and cPKCβII activation was elucidated in this study.

METHODS

Data sources
A total of 30 CHD patients without AS and 30 CHD patients...
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$_2$S 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$_2$S concentration until the reading is stable. H$_2$S concentration was calculated according to a calibration curve obtained with known H$_2$S concentrations in a range between 5 and 100 µmol/L, utilizing the H$_2$S 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 mM/L Tris-Cl, pH 7.5, containing 2 mM/L dithiothreitol, 2 mM/L EDTA, 1 mM/L EGTA, 5 g/ml each of leupeptin, aprotinin, pepstatin A and chymostatin, 50 mM/L potassium fluoride, 50 mM/L okadaic acid, 5 mM/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 mM/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/χ² 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/m²)            | 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 (µmol/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 |
| RASl, 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 H₂S level in CHD patients was significantly lower than the control group (P < 0.05). Meanwhile, the plasma H₂S 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 H₂S (r = −0.970, P = 0.000). No correlation with age, gender, dialysis duration, serum TG, TC, smoking, and hypertension [Table 2].

**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. 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%. 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. Subsequent investigations elucidated abnormal atherosclerotic pathology in patients with CKD may be classified as AS, arteriosclerosis, and vascular calcification. 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.

Hydrogen sulfide is an endogenous gas with modulating actions, which has been proposed as an antioxidant due to its ability to protect against oxidative stress and to react with oxidized thiols forming hydrodisulfide. H₂S is synthesized from L-cysteine by two pyridoxal-5’-phosphate-dependent enzymes, cystathionine γ-lyase (CSE) or cystathionine β-synthase (CBS). CBS activity is predominant in H₂S synthesis in the central nervous system whereas CSE is the major H₂S synthesis enzyme in the cardiovascular system. A variety of studies have shown the physiological and pathophysiologic functions, including regulation of BP renal damage, and neurodegenerative diseases. H₂S can decrease the cardiovascular risk through protecting the L-NAME-induced hypertensive rats against liver injury via...
The deficiency of H\(_2\)S was involved in the pathogenesis of AS,
and the CSE/H\(_2\)S 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\(_2\)S 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\(_2\)S in CHD patients and this decrease was more
significant in CKD patients with AS, which prompted that
decrease of H\(_2\)S 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.\(^{[34]}\)
cPKC\(\beta\)II 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 cPKC\(\beta\)II in mice results in a
significant reduction in the progression of AS.\(^{[10]}\) Moreover,
there is an increasing interest in developing cPKC\(\beta\)II
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 cPKC\(\beta\)II 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 cPKC\(\beta\)II inhibitor in combination with
SOC, can help reduce fat accumulation, improve glucose
tolerance, decrease hepatosteatosis and suppress foam cell
formation.\(^{[38]}\) Meanwhile, cPKC\(\beta\)II 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, cPKC\(\beta\)II
specific inhibitors have been clinically investigated on
AS‑associated diseases.\(^{[40]}\)

Table 2: Correlation coefficients for cPKC\(\beta\)II and other
variables in CHD + AS patients

| Variables          | \(r\)  | \(P\)   |
|--------------------|--------|---------|
| H\(_2\)S            | −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\(_2\)S: Hydrogen sulfide; cPKC\(\beta\)II: Conventional
protein kinase C\(\beta\)II; CHD: Chronic hemodialysis; AS: Atherosclerosis.

Figure 2: The membrane translocation of conventional protein kinase C\(\beta\)II (cPKC\(\beta\)II) 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 cPKC\(\beta\)II membrane translocation in CHD + AS group increased significantly compared with CHD
group (\(P < 0.05\) vs. Control group; \(P < 0.05\) vs. CHD group).

A study from Pan et al. prompted that H\(_2\)S 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.\(^{45}\) Similarly, in our present study, we found that the cPKC\(\beta\)II activation was negatively correlated with plasma H\(_2\)S in CHD + AS patients.

In summary, these findings in this study suggest a possible linkage between H\(_2\)S metabolism and cPKC\(\beta\)II activation, which may contribute to the development of UAAS in CHD patients.

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