The compensatory enrichment of sphingosine -1-phosphate harbored on glycated high-density lipoprotein restores endothelial protective function in type 2 diabetes mellitus

Xunliang Tong1, Pu Lv1, Anna V Mathew3, Donghui Liu4, Chenguang Niu2, Yan Wang4, Liang Ji2, Jizhao Li2, Zhiwei Fu2, Bing Pan2, Subramaniam Pennathur3, Lemin Zheng2* and Yining Huang1*

Abstract

Background: Glycation of high-density lipoprotein (HDL) decreases its ability to induce cyclooxygenase-2 (COX-2) expression and prostacyclin I-2 (PGI-2) release in endothelial cells. Whether lipid content of HDL, especially sphingosine-1-phosphate (S1P), plays any specific role in restoring the protective function of HDL in type 2 diabetes mellitus (T2DM) is still unknown.

Methods and results: Immunochemical techniques demonstrated that glycated HDL loses its protective function of regulating COX-2 expression compared with diabetic HDL. We proved that the lipid content, especially phospholipid content differed between diabetic HDL and glycated HDL. Levels of HDL-c-bound S1P were increased in T2DM compared with control subjects as detected by UPLC-MS/MS (HDL-c-bound S1P in control subjects vs. T2DM: 309.1 ± 13.71 pmol/mg vs. 382.1 ± 24.45 pmol/mg, P < 0.05). Additionally, mRNA levels of S1P lyase enzymes and S1P phosphatase 1/2 were decreased in peripheral blood by real-time PCR. Antagonist of S1P receptor 1 and 3 (S1PR1/3) diminished the functional difference between apoHDL&PL (HDL containing the protein components and phospholipids) and diabetic apoHDL&PL (diabetic HDL containing the protein components and phospholipids). With different doses of S1P reconstituted on glycated HDL, its function in inducing the COX-2 expression was restored to the same level as diabetic HDL. The mechanism of S1P reconstituted HDL (rHDL) in the process of regulating COX-2 expression involved the phosphorylation of ERK/MAPK-CREB signal pathway.

Conclusion/Significance: S1P harbored on HDL is the main factor which restores its protective function in endothelial cells in T2DM. S1P and its receptors are potential therapeutic targets in ameliorating the vascular dysfunction in T2DM.

Keywords: Sphingosine 1 phosphate, High density lipoprotein, Type 2 diabetes mellitus, Cyclooxygenase-2, Endothelial cells

* Correspondence: zhengl@bjmu.edu.cn; ynhuang@sina.com

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**Introduction**

Diabetes mellitus is a systemic metabolic disease and a major risk factor for the development of atherosclerosis [1,2]. Hyperglycemia causes alterations in lipid metabolism which bring about a series of adverse effects, including enhanced HDL clearance, decreased apoA-1 transcription and accelerated HDL glycation [3,4]. These dramatically altered lipid metabolic changes can promote atherosclerosis [5,6].

Native HDL has protective role in inducing cyclooxygenase-2 (COX-2) expression and prostacyclin I-2 (PGI-2) release in endothelial cells [7]. PGI-2 is the major COX catalyzed product which is metabolized from arachidonic acid (AA) and plays a protective role in the macrovascular endothelium [7,8]. Previous studies have shown that the lipid component of HDL can induce COX-2 expression and PGI-2 release, especially sphingosine-1-phosphate (S1P) [9]. S1P is primarily carried on HDL in plasma and has many biological functions, such as ability to promote vasodilation, vasoconstriction, and angiogenesis, protect against ischemia/reperfusion injury, and inhibit/reverse atherosclerosis [10]. The level of S1P is controlled by two pathways, dephosphorylation of S1P regenerating sphingosine by S1P-specific phosphatases (SGPP), and the other is the irreversible cleavage by sphingosine-1-phosphate lyase (SPL) [11-14].

Non-enzymatic glycation is a common modification that occurs in type 2 diabetes mellitus (T2DM), which could impair protein function [15,16]. Previous studies showed that non-enzymatic glycation of HDL impairs its anti-inflammatory function and vascular protective effects [17-19]. Diabetic HDL could also down-regulate expression of SR-BI causing a dysfunction in proliferation and migration of endothelial cells [20]. Previous studies have shown alteration of sphingosine metabolism in diabetes mellitus [21-23], so in this study, we focused on the effect of the lipid component of HDL after glycation impairment. We recently proved that HDL-associated S1P was increased in T2DM than healthy volunteers [24]. In this work, we attempt to elucidate the mechanism of S1P’s protective function in inducing COX-2 expression and PGI-2 release.

**Methods**

**Ethical approval**

Healthy control subjects and patients with type 2 diabetes were recruited following informed consent. Human umbilical vein endothelial cells (HUVECs) were obtained by collagenase digestion of umbilical cords which were donated by the volunteers after written informed consent. The study was approved and supervised by the Institutional Review Board and Ethics Committee of Peking University First Hospital (Beijing, China).

**Chemical agents**

S1P, C17-D-erythro-sphingosine-1-phosphate (C17-sph) and the antagonist of S1PR1 and S1PR3 were purchased from Avanti polar lipids (Alabama, USA). The antibodies for western blot against phospho-extracellular regulated kinase (ERK) 1/2 and cAMP-response element binding protein (CREB) were purchased from Cell Signal Technology, Danvers, MA. The antibodies of phospho-p38 mitogen-activated protein kinase (MAPK), p38 MAPK and β-actin were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Antibody against COX-2 and competitive enzyme immunoassay kit for 6-keto PGF1α were from Cayman Chemical (Michigan, IL). Horseradish peroxidase (HRP)-goat-anti-rabbit IgG and HRP-goat-anti-mouse IgG were purchased from MBL (Nagoya, Japan). Endothelial cell medium (ECM) was purchased from ScienCell Research Laboratories (Carlsbad, CA).

**Subjects**

Healthy volunteers and patients underwent physical examination, laboratory tests and ultra-sound examination of the large arteries. The patients were all first diagnosed with T2DM in accordance with international standards; fasting plasma glucose (FPG) ≥ 7.0 mmol/L and glycated hemoglobin (HbA1c) greater than 6.5%. The patients were all first diagnosed in the hospital before initiation of treatment. The healthy subjects had no family history of diabetes, and they had normal FPG level and normal glucose tolerance. Allowing the criteria above, 15 T2DM and 15 healthy patients were recruited. The detailed information of the recruited patients and healthy volunteers has been described in a previous study [24].

**Cell culture**

Human umbilical vein endothelial cells (HUVECs) were obtained by collagenase treatment of umbilical cord vein as described previously [25]. Cells were cultured on gelatin-coated dishes and propagated in endothelial cell medium supplemented with 5% FBS and endothelial cells were grown at 37°C in an incubator with humidified air containing 5% CO2. HUVECs were harvested when cells reached 70–80% confluent and passages three to fifty were used.

**Blood collection and HDL isolation**

The fresh blood from T2DM and healthy volunteers was drawn after overnight fasting into EDTA-Na2 vacuum tubes. Plasma was separated by centrifuging at 2,500 rpm at 4°C for 15 minutes. HDL (1.063-1.210 g/ml) was isolated by ultracentrifugation as described previously [26]. HDL2 (1.063 < d < 1.125 g/ml) and HDL3 (1.125 < d < 1.21 g/ml) were isolated by sequential ultracentrifugation as previously described [27]. HDL sub fractions were dialyzed against saline/EDTA (150 mM NaCl, 300
Modification, selected delipidation and reconstitution of HDL

HDL was incubated in glucose/phosphate-buffer saline (PBS) buffer with butylated hydroxytoluene (BHT) (the final concentration of glucose is 25 mmol/L) at 37°C for 7 days in vitro [17]. During this procedure, the oxidation levels of modified HDL were measured (Additional file 1: Figure S1). After HDL was dialyzed with PBS, glycated HDL was used for cell experiments and reconstitution. Selective delipidation was achieved as described previously. HDL was agitated with di isopropyl ether in a ratio of 1:2 (vol/vol) at 4°C for 24 h [28]. After extraction, the mixture was centrifuged at 2,000 rpm for 5 minutes to separate the aqueous and organic phase. Apo HDL&PL to (HDL containing the protein components and the phospholipids) was collected in the aqueous phase and PL-depleted HDL-lipids (the lipid component of HDL except for the phospholipids) in the organic phase. Alternatively, HDL was incubated in a mixture of butanol and di isopropyl ether (vol/vol, 40:60) in a ratio of 1:2 (vol/vol) for 30 minutes at the room temperature. After centrifugation we collected apoHDL (HDL containing the protein components only) in aqueous phase and HDL-lipids (all lipid component of HDL) in organic phase. ApoHDL&PL and apoHDL were filtered before use. Reconstituted HDL (rHDL) was made by adding S1P to glycated HDL at the desired concentration and then mixed by rotation overnight at 4°C for further usage [29]. The levels of S1P used for reconstituting were nearly equal to the S1P we detected on reconstituted HDL (Additional file 1: Figure S2).

S1P extraction and detection by UPLC-MS/MS

UPLC-MS/MS technique was employed to measure the levels of HDL-associated S1P as previously described. Serum samples mixed with internal standard C17-sph (50 μl of 1000 μg/L) was precipitated by methanol at the volume ratio of 1:4. After centrifugation at 12,000 rpm for 15 minutes, the supernatant was collected for UPLC-MS/MS analysis performed by a Waters ACQUITY UPLC™ system as described previously [24]. Waters ACUITY UPLC BEH Phenyl column (1.8 μm; 2.1 mm × 100 mm) was selected for chromatographic separation. The injection volume was 5 μL.

Methanol (A) and 0.5% formic acid in ultrapure water (B) were used as mobile phases. The gradient started at 10% A and then increased linearly to 60% in 6 minutes, and then to 100% at 6 minutes and kept for 2 minutes, followed by a decrease to initial conditions of 10% A and held for 2 minutes to allow for equilibration. The flow rate was 0.3 mL/min. The column was maintained at 40°C, and the sample room temperature was 10°C.

Real-time PCR assay for SPL and SGPP1/2

Real-time PCR was performed to determine mRNA levels of SPL and SGPP1/2 from peripheral blood of the patients and healthy volunteers. Total RNA was extracted using the TRIzol reagent (Invitrogen, USA) and reverse transcription was performed using an RT-PCR kit (TransGen Biotech, China). Real-time experiments were conducted on a DNA Engine Opticon System (MJ research Inc, USA) using SYBR Green PCR Master Mix kit in triplicate specific primers. The sequences of primers to determine the expression of the target gene were as follows: SPL [5’-CTTGATG CACTTCGGTGAGA-3’ (forward); 5’-TCCACCCCTTAG CA GT CATC-3’ (reverse)], SGPP1 [5’-ACGGC CATCCCC ATTTCT-3’ (forward); 5’-AGGAATCCAGCAATAATATC CAG-3’ (reverse)], SGPP2 [5’-gTATTTACTCATGGTTC AAGGTG-3’ (forward); 5’-GTGAGGTTAACACACTGT TAAGG-3’ (reverse)] and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [5’-CGGAGTCAACGGATTTGG TCGTAT-3’ (forward); 5’-AGCCTTCTCCATGGTGTTGA AGAC-3’ (reverse)]. The PCRs consisted of 5 min at 95°C followed by 40 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 56°C and a primer extension for 30 sec at 72°C. The comparative CT method was used to quantify the expression of SPL, SGPP1 and SGPP2 using GAPDH as the normalized control.

ELISA: quantitation of 6-keto PGF1α

PGI-2 is non-enzymatically hydrated to 6-keto PGF1α and estimation of systemic PGI-2 production has often been assessed by measurement of 6-keto PGF1α [30]. The cell culture supernatants were collected after centrifugation at 3000 rpm for 15 minutes. For accurate measurement of PGI-2 production, 6-keto PGF1α in cell culture supernatants were determined using enzyme immunoassay kit (Cayman Chemical). The final results were normalized to cell protein concentration.

Western blotting and EMSA

Western blot analysis was performed as described previously [31], cells were washed twice with cold 1X PBS and lysed on ice for 30 min in lysis buffer. The lysates were subjected to centrifugation at 12,000 rpm for 15 min at 4°C and the supernatant was utilized for analysis. Protein concentrations were determined using BCA method. The boiled samples were loaded on Ready SDS-10%
PAGE gels and used for Western blot analysis with the protein-specific antibody. HRP-labeled secondary antibody was used for detection of signal by electrochemiluminescence from Pierce (California, USA).

DNA-protein binding reactions were performed by incubating nuclear extracts with specific CREB-CIE (cis-inducible element) DNA binding. Polyclonal anti-CREB antibody was added to the reaction mixture containing the labeled probe. EMSA was performed as previously described [32].

For EMSA assay, 10 μg nuclear protein was used with 15 fmol of HRP-end-labeled double stranded oligonucleotides in mixed nuclear extraction from HUVEC after treated with HDL as described above. The sequence of oligonucleotide containing CREB was: 5'-AGA GAT TGC CTG ACG TCA GAC AGC TAG-3. Oligonucleotides were end-labeled with HRP polynucleotide kinase and purified on G-50 columns (Roche Diagnostics). The DNA binding reaction of HRP labeled double-stranded oligonucleotides was performed at room temperature for 20 min, according to the manufacturer’s protocol. Electrophoretic mobility shift assays were performed with the Light Shift Chemiluminescent Kit from Pierce, according to the manufacturer’s recommendations.

Statistical analyses
All experiments were repeated in triplicate if not mentioned. Data are presented as mean ± SEM unless indicated otherwise. Differences were compared with two-tailed Student’s t-test or one-way ANOVA using GraphPad Prism software. p < 0.05 was considered statistically significant.

Results
Lipid component of diabetic HDL improved the up-regulation of COX-2 expression and PGI-2 release in endothelial cells
To test whether the glycation modification impaired HDL function in endothelial cells, we used native HDL and non-enzymatically glycated HDL treated with HUVECs at the final concentration of 30 μg/ml for 6 hours. Glycated HDL failed to induce COX-2 expression (Figure 1A). Compared with glycated HDL, the diabetic HDL showed significantly increased induction of COX-2 expression (Figure 1B). To eliminate the differences in lipid component, delipidated HDL was used to treat HUVECs and the delipidated apoHDL regulated COX-2 expression slightly more than delipidated diabetic apoHDL. Both apoHDL and diabetic apoHDL partially lost the ability in up-regulating COX-2 expression (Figure 1C), which suggests that such effects are in part mediated by lipid component instead of protein. The selectively delipidated HDL, apoHDL&PL were used to incubate with HUVECs at the final protein concentration of 30 μg/ml for 6 hours. Diabetic apoHDL &PL restored the effects of inducing COX 2 expression compared with apoHDL&PL (Figure 1D), which implies that the phospholipid (PL) component was essential for these effects.

S1P levels are increased in lipid component of HDL in diabetics compared to controls
To investigate the S1P level in plasma and HDL, we examined samples from 15 T2DM patients and control subjects using UPLC-MS/MS. As S1P in plasma was mainly bound to HDL-C, we normalized the levels of total plasma S1P to plasma HDL-C, which gave us the level of HDL-bound S1P. There was an increased HDL-C-normalized level of S1P in diabetes mellitus compared with control subjects (Figure 2A, control subjects vs. T2DM: 309.1 ± 13.71 pmol/mg vs. 382.1 ± 24.45 pmol/mg,
S1P harbored on diabetic HDL (Y axis) were decreased with the increasing levels of HbA1c (Additional file 1: Figure S3). To investigate the mechanism for increased level of S1P from diabetic HDL, the expression of related enzymes that degenerate S1P was examined. The mRNA levels of SPL and SGPP 1/2 from peripheral blood was detected by the method of real-time PCR. The level of SPL was decreased 1.6 fold and the levels of SGPP1 and SGPP2 were also decreased 4.8 fold and 15.7 fold separately in diabetes compared with control subjects (Figure 2C).

Figure 2 Levels of S1P and its related enzymes were detected by UPLC-MS/MS and real-time PCR. Levels of HDL-bound S1P were significantly increased in diabetes mellitus than control subjects. A-B: S1P level was normalized by HDL-c in plasma between control subjects and T2DM (A), levels of diabetic HDL3-associated S1P were increased compared with native HDL3-associated S1P (B). *p < 0.05 vs. healthy group. The levels of mRNA involved in S1P metabolism was measured by real-time PCR. C: The mRNA level of SPL and SGPP1/2 was detected in T2DM and control subjects. Mann–Whitney U test. Bars show medians. * p < 0.05.

Reconstitution of S1P on glycated HDL was as effective as diabetic HDL in inducing COX-2 expression and PGI-2 release
We used different doses of free S1P for binding on HDL particles incubated with HUVECs and expression of COX-2 was induced by the reconstituted HDL in a dose-dependent manner (Figure 4A). The reconstitution of S1P on glycated HDL restored the ability of inducing COX-2 expression and PGI-2 release and was dose dependent (Figure 4B). These results show that S1P reconstituted on HDL can reverse the loss of function caused by glycation modification.

Reconstituted HDL with addition of S1P on glycated HDL activates the ERK/MAPK pathways
The signal transduction activated by COX-2 expression involves the ERK/MAPK-CREB pathway (29). Three kinds of HDL, native HDL, glycated HDL (G-HDL) and the reconstituted HDL (rHDL, S1P added on glycated HDL) were used to treat HUVECs respectively. The glycated HDL
HDL was utilized to generate rHDL to reflect the increased glycation to HDL in subjects with T2DM. rHDL showed similar function when compared with native HDL, while the G-HDL failed to activate the signaling pathway. rHDL showed the most effect in activation of the pathway at each tested time point (Figure 5A, B and C, P < 0.01), suggesting a selective effect of S1P in mediating these effects. The phosphorylation was triggered by the three types of HDL starting at 5 minutes and reached the peak at 15 minutes, and returned to baseline at 60 minutes. The effect of rHDL in activation of the phosphorylation of ERK and MAPK was reduced by the VPC23019 (Figure 5D, E and F) suggesting that this effect was mediated by the S1P receptors.

Reconstituted HDL enhances the transcription factor CREB binding on the cis-inducible element (CIE) DNA

Since ERK1/2 and MAPK were the modulators of the CREB protein, we tested whether the three kinds of HDL enhanced the nuclear transcription factor CREB activity or not. rHDL and native HDL markedly increased CREB phosphorylation, whereas G-HDL had a considerably weaker effect and antagonist of S1PR1 and S1PR3 reduced effects of rHDL (Figure 6A). As shown in Figure 6B, reconstituted HDL enhanced more transcription factor binding on the CIE DNA than the other two forms of HDL. The binding of CREB to the CIE sequence disappeared with 50-fold and 100-fold excess of unlabeled CIE DNA (cold probe), confirming the specificity of CIE DNA binding. CREB binding with CIE as CREB/CIE DNA complex got abolished when anti-CREB antibody was added in the system. These results demonstrate that extra S1P loading on glycated HDL induced more transcription factor CREB binding on its CIE DNA complex.

Discussion

Diabetes mellitus is associated with HDL dysfunction which accelerated atherosclerosis, in which HDL particle undergoes diverse structural modifications resulting in significant changes in its composition and function [6]. In this study, we investigated one of the most common modifications, glycation and a well-known bioactive lipid molecule, S1P.
Previous studies have shown that non-enzymic glycation was a common modification that appeared in T2DM and this modification impaired HDL’s anti-inflammatory and anti-oxidant roles [33]. In this study, we explored the mechanism involved in the effect of S1P in repairing the function of glycated HDL, like inducing COX-2 expression and PGI-2 release. We have previously shown that the level of diabetic HDL-associated S1P is increased compared with native HDL [25]. As the following work of that research, we found out that in the T2DM group, the level of S1P harbored on HDL in T2DM patients decreased with the elevated level of HbA1c. So we assumed that higher level of S1P on HDL could serve as protective effects to the vascular endothelial, but with the progress of T2DM, the level of S1P was decreased and the function of it was also diminished. At the same time, we tried to figure out the potential mechanism involved in the increased level of S1P associated with diabetic HDL [25]. The explanation of the increased level of diabetic HDL-associated S1P was related to its
levels of expression of key elimination enzymes. S1P is generated by SphK1/2 from sphingosine and eliminated by dephosphorylation and lyase reactions [34]. The dephosphorylation of S1P is catalyzed by the actions of S1P-specific phosphatases, as well as by enzymes of the nonspecific lipid phosphate phosphatase family (LPPs). Each of these enzymes regenerates sphingosine which can be rapidly re-phosphorylated, thereby restoring S1P levels. In contrast to the phosphatases, SPL catalyzes an irreversible cleavage reaction that provides global control over circulating S1P levels and tissue S1P pools [14,15]. As the final enzyme in the sphingolipid degradation pathway, SPL controls the only exit point for sphingolipid intermediates and their flow into phospholipid metabolism [34]. Thus, in addition to regulating S1P levels, SPL is the gatekeeper of a critical node of lipid metabolic flow. The SPL plays the key role in regulation of the S1P level in plasma and recruitment back to sphingosine and thereby influencing S1P-dependent biological and pathological processes [24]. In our study, we found SPL activity is decreased which perhaps leads to the increased level of HDL-associated S1P increased in T2DM [25]. Previous studies were shown that S1P was preferentially associating with HDL3 [35]. In our work, we confirmed that the increasing the level of HDL3-associated S1P restores its function similar to what is observed with HDL from diabetic subjects. This suggests that perhaps in T2DM the increased levels of S1P may be a compensatory protective mechanism.

The role of S1P in atherosclerosis is complex, as it has both pro- and anti-atherosclerotic properties as shown in previous studies [36,37]. The endothelial system sets up the first barrier for the blood vessels and HDL with S1P plays the important role in this protective procedure. S1P receptors are G-coupled receptors located on endothelial membrane where S1PR1 and S1PR3 are mainly located. So the increased level of S1P occurring in diabetic HDL3 may have vascular protective effects. The accumulation of the lipids on HDL, especially S1P, compensates the functional loss and may exert the role of protection by regulation of COX-2 expression and activation of ERK/MAPK-CREB pathway.

In addition to increased risk of stroke, myocardial infarction, and peripheral vascular disease, diabetics suffer from a particularly aggressive form of atherosclerosis with greater in-hospital mortality following myocardial infarction, a higher incidence of heart failure and stroke [6]. The prevention of complications needs more focus.

**Conclusions**

The current study demonstrates that the increasing level of S1P occurring in diabetic HDL3 have vascular protective effects. The accumulation of the lipids on HDL, especially S1P, compensates the functional loss and may exert the role of protection by regulation of COX-2 expression and activation of ERK/MAPK-CREB pathway. Such increased S1P concentration possibly results from the down-regulation of its degeneration enzymes (SPL and SGPP).

Our work sheds more light on the intrinsic mechanism behind the protective effects of HDL in diabetic disease thus opening doors for new therapeutic targets. Since currently available drug use in clinical practice has not provided us with appreciable results, new targets and insights are needed as soon as new disease mechanisms are recognized. The S1P pathway activation provides a potential site for future drug design.
Additional file

Additional file 1: Figure S1. Detection of MDA level and PON1 activity in N-HDL and G-HDL by spectrophotometry. The differences of MDA level and PON1 activity in the two groups have no statistical significance. Figure S2. The level of S1P reconstituted on HDL (30 mg/ml) was detected by the method of UPLC-MS (A). The levels of S1P were reconstituted were nearly equal to the S1P we detected on reconstituted HDL. Figure S3. In the T2DM group, levels of S1P harbored on HDL (Y axis) were decreased when the levels of HbA1c (X axis) increased. The correlation coefficient R was 0.7574, P < 0.001.

Abbreviations
T2DM: Type2 diabetes mellitus; HDL: High-density lipoprotein; rHDL: Reconstituted HDL; S1P: Sphingosine-1-phosphate; COX-2: Cyclooxygenase-2; PG-2: Prostacyclin I-2; HUVECs: Human umbilical vein endothelial cells; S1PR1: S1P receptor 1; S1PR3: S1P receptor 3; apoHDL&PL: HDL containing the protein components and the phospholipids; SGPP: S1P-specific phosphatases; SPL: Sphingosine-1-phosphate lyase.

Competing interests
The authors declare they have no competing interests.

Authors’ contribution
As to the contribution of each author, XT, LZ and YH designed the study, analyzed and interpreted the results, and drafted the manuscript; PLV, DL, CN, YW, LJ, JL, ZF made substantial contributions to performing the experimental protocol; AVM and SP were involved in revising the manuscript critically for important intellectual content. All authors participated in the discussion and interpretation of the results and in the final approval of the manuscript submitted.

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Author details
1Department of Neurology, Peking University First Hospital, Beijing 100034, China. 2The Institute of Cardiovascular Sciences and Institute of Systems Biomedicine, School of Basic Medical Sciences, and Key Laboratory of Molecular Cardiovascular Sciences, Ministry of Education, Key Laboratory of Cardiovascular Molecular Biology and Regulatory Peptides, Ministry of Health, Peking University Health Science Center, Beijing 100019, China. 3Department of Internal Medicine, University of Michigan, Ann Arbor; Michiigan 48109, USA. 4Division of cardiology, Zhongshan Hospital affiliated to Xiamen University, Xiamen heart center, Xiamen, Fujian Province 361004, China.

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