Total plasma sulfide in mild to moderate diastolic heart dysfunction

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ABSTRACT

Background. The early pathophysiological mechanisms of diastolic dysfunction are not understood well. Hydrogen sulfide is an important endogenous gaseous transmitter that can influence heart remodeling. The aim was to determine total plasma sulfide (TPS) levels, as a surrogate marker of hydrogen sulfide, in patients with mild diastolic dysfunction.

Methods. Total plasma sulfide and N-terminal pro brain-type natriuretic peptide (NT-proBNP) levels were determined in ambulatory patients with arterial hypertension or diabetes mellitus and echocardiographically mild to moderate diastolic dysfunction.

Results. Twenty-four patients were included: nine with normal diastolic function (Grade 0), eight with an impaired relaxation pattern (Grade 1), and seven with a pseudo-normalized pattern (Grade 2). TPS levels were highest in patients with normal diastolic function (Grade 0), and lowest in patients with Grade 2 diastolic dysfunction, with this difference between Grade 0 and Grade 2 showing statistical significance (p = 0.017). NT-proBNP levels showed the reverse behavior, with this difference again showing statistical significance (p = 0.042).

Conclusions. Total plasma sulfide levels decrease with worsening of diastolic function from normal to moderate diastolic dysfunction.

Key words: total plasma sulfide, hydrogen sulfide, arterial hypertension, diastolic dysfunction, echocardiography

INTRODUCTION

Heart failure with preserved ejection fraction is a new condition that has only been described in recent years. (1) One of its defining criteria is diastolic dysfunction. It has gained a lot of attention since its incidence has been rising and there is still no solid guidelines for treatment or prevention. There remains a huge gap in our understanding of the pathological and pathophysiological processes during the primary and advancing stages of diastolic dysfunction. The key event is myocyte hypertrophy and collagen deposition, which causes diastolic relaxation problems. Insufficient diastolic relaxation results in higher left ventricular filling pressure, left atrial stretch, and pulmonary congestion. Clinically, all of these represent the exercise dyspnea and signs of pulmonary congestion seen in cases of advanced heart failure. (2)

Hydrogen sulfide (H2S) is a gas that has the characteristic odor of rotten eggs, and it is generally known for its toxicity. (3) H2S is toxic because it binds to cytochrome c oxidase, and therefore inhibits the mitochondrial respiratory chain. As well as showing toxicity, H2S has an important role in signaling at the cellular level. H2S can modulate vascular tone and neuronal function, and it can also be cryoprotective during ischemia. (4) H2S has recently emerged as an important gaseous transmitter in mammals, along with nitric oxide and carbon monoxide. (5)

In mammals, H2S is synthesized from the sulfur-containing amino acid L-cysteine through the activities of either cystathionine-β-synthase or cystathionine-γ-lyase, both of which require vitamin B6 as a cofactor. (5) A further mitochondrial enzyme has been described recently to also synthesize H2S: 3-mercaptopyruvate sulfurtransferase. (6) In conjunction with cysteine aminotransferase, this enzyme also contributes significantly to the generation of H2S. All three of these enzymes are expressed in smooth muscle and endothelium. Since H2S shows not only complex production, but also has a complex influence on various tissues, it has been studied extensively in recent years. Although H2S is a gas, it is very short-lived due to its dissolution to form a weak acid: HS− and S2− (although S2− is negligible at physiological pH). Here, we will use the term H2S only for the gaseous form, and in all other instances, the term sulfide will refer to the combined gas and anions, as the total plasma sulfide (TPS). (7) Due to the rapid turnover of H2S, it is difficult to obtain meaningful measurements under various clinical conditions. We therefore determined TPS, which comprises H2S plus the dissolved and protein-bound sulfide.

The aim of the current study was to determine the levels of TPS and N-terminal pro-brain-type natriuretic peptide (NT-proBNP) in ambulatory hypertensive or diabetic patients with mild to moderate diastolic dysfunction and no clinical signs of pulmonary congestion.
**MATERIALS AND METHODS**

**Study population**

Twenty-four consecutive patients who were referred for ambulatory echocardiographic examination in Celje General and Teaching Hospital (Celje, Slovenia) and showed preserved left ventricular systolic ejection fraction were included in this study, between May 2009 and May 2011. Informed consent was obtained from each patient. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki, revised in 2010, as reflected in the prior approval by the Institutional Human Research Committee (N° 117bis/01/10). The study protocol was also approved by the National Ethical Human Research Committee.

The exclusion criteria included age <18 years, valvular heart disease, congestive heart failure, liver failure, hypothyroidism or hyperthyroidism, pheochromocytoma, acute infection, malignancy, and psychiatric disorders that limited cooperation. The additional inclusion criteria included systemic arterial hypertension and/or diabetes mellitus type 1 or type 2, as documented and treated for ≥5 years. All of the patients had to be in New York Heart Association (NYHA) class I or II heart failure without signs of pulmonary congestion.

**Estimation of diastolic function**

Echocardiographic studies were performed (GE Vivid 7 or GE Vivid 6; GE Healthcare, USA), with trans-thoracic echocardiograms completed according to the laboratory protocol. All of the patients underwent a standard echocardiographic study to exclude other abnormalities. Echocardiographic images were read by three blinded investigators (N.G.P, D.K., M.P.) for the re-measurement of all of the relevant parameters. These included ejection fraction, end diastolic volume (estimated using the Teicholz and Simpson method), (8) peak early and atrial velocities of mitral inflow, early mitral inflow deceleration time, and septal and lateral mitral annular velocities (e’). Where possible, the mean for each measurement was taken over multiple cardiac cycles.

The diastolic function grading was based on the relevant guidelines. (9) In cases where the parameters were non-congruent, the diastolic dysfunction grade was established as that with the highest number of characteristic parameters, with the assumption of equal weighting. Thus, the patients with normal diastolic function were classified as Grade 0. The patients were classified as having mild diastolic dysfunction (Grade 1) according to: mitral early/atrial ratio, <0.8; deceleration time, >200 ms; isovolumic relaxation time, 100 ms; predominantly systolic for pulmonary venous flow (i.e., systolic > diastolic); annular e’, <8 cm/s; and mean E/e’ ratio, <8 (septal and lateral). The patients were classified as having moderate diastolic dysfunction (Grade 2) according to: mitral early/atrial ratio, 0.8 to 1.5 (pseudonormal), which decreased by >50% during the Valsalva maneuver; annular e’, <8 cm/s; and mean E/e’ ratio, 9 to 12.

**Total plasma sulfide and NT-proBNP measurements**

Total plasma sulfide and NT-proBNP were measured in blood samples taken from the patients at the time of their echocardiographic study. TPS was measured by a modified spectrophotometric method, (10) which was first described in 1949 by Fogo. (11) This method was further refined in 1965 by Siegel, (12) and has been used in other studies afterwards. (13-17) Immediately after collection, blood samples were briefly centrifuged at 3000 rpm for 10 min at 4°C to obtain the plasma. Two hundred microliters of each plasma sample was mixed with 100 μL of a pre-prepared solution of 10% (v/v) trichloroacetic acid, and 60 μL of 1% (w/v) zinc acetate, to trap any dissolved H2S. The mixture was then frozen at -20°C until further analysis. Upon defrosting of the samples, 40 μL 20 μM N, N-dimethyl-p-phenylenediamine sulfate prepared in 7.2 M HCl, and 40 μL 30 μM FeCl3 prepared in 1.2 M HCl, were added. After vortexing, these samples were incubated for 20 min at room temperature to allow the color reaction to develop, and then they were centrifuged at 9000 rpm for 5 min at 4°C, to remove the precipitate. The absorbance at 670 nm was then determined spectrophotometrically (Epoch microplate spectrophotometer, Biotek, VT, USA) for the resulting blue-colored supernatants. The TPS concentrations of the samples were then calculated from the absorbance calibration curve of known Na2S concentrations. To ensure accurate measurements, all of the samples were analyzed in triplicate, with the data expressed as median [range]. The NT-proBNP levels were determined using electrochemiluminescence immunoassay (Elecys; Roche Diagnostics, Switzerland) according to manufacturer protocol.

**Disccussion**

The present study shows lower TPS levels in the plasma of patients with moderate diastolic dysfunction compared to those with normal diastolic function. Conversely, the NT-proBNP levels increased with increasing severity of diastolic dysfunction. Diastolic dysfunction is a pathophysiological concept that is defined by decreased relaxation of the left ventricular myocardium.
Table 1. Demographic and clinical characteristics of the patients included in this study.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Datum (n = 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>54 [32, 77]</td>
</tr>
<tr>
<td>Gender (female/male)</td>
<td>12/12</td>
</tr>
<tr>
<td>Diseases</td>
<td></td>
</tr>
<tr>
<td>Arterial hypertension [n %]</td>
<td>17 (70)</td>
</tr>
<tr>
<td>Diabetes mellitus [n %]</td>
<td>13 (54)</td>
</tr>
<tr>
<td>Therapies</td>
<td></td>
</tr>
<tr>
<td>Angiotensin-converting enzyme inhibitor [n %]</td>
<td>13 (54)</td>
</tr>
<tr>
<td>Beta-blocker [n %]</td>
<td>7 (30)</td>
</tr>
<tr>
<td>Angiotensin receptor blocker [n %]</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Diuretic [n %]</td>
<td>6 (25)</td>
</tr>
<tr>
<td>Spironolactone [n %]</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Other anti-hypertensive therapy [n %]</td>
<td>6 (25)</td>
</tr>
<tr>
<td>Physiology</td>
<td></td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>65</td>
</tr>
<tr>
<td>Systolic arterial pressure (mmHg)</td>
<td>134 ±17</td>
</tr>
<tr>
<td>Diastolic arterial pressure (mmHg)</td>
<td>74 ±14</td>
</tr>
<tr>
<td>Laboratory analyses</td>
<td></td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>79 ±21</td>
</tr>
<tr>
<td>NT-proBNP (ng/L)</td>
<td>319.3 ±786</td>
</tr>
</tbody>
</table>

Table 2. Echocardiographic data of the patients included in this study.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Unit</th>
<th>Datum (n = 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic function</td>
<td></td>
<td></td>
</tr>
<tr>
<td>End diastolic volume (Simpson)</td>
<td>mL</td>
<td>77 ±24 [63-78]</td>
</tr>
<tr>
<td>Left ventricular ejection fraction (Simpson)</td>
<td>%</td>
<td>67 ±11 [60-73]</td>
</tr>
<tr>
<td>(Teicholz)</td>
<td>%</td>
<td>69 ±9 [63-76]</td>
</tr>
<tr>
<td>Diastolic function</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early peak velocity of mitral inflow A</td>
<td>m/s</td>
<td>0.9 ±0.2 [0.7-1.1]</td>
</tr>
<tr>
<td>Late peak velocity of mitral inflow E</td>
<td>m/s</td>
<td>0.87 ±0.12 [0.78-0.91]</td>
</tr>
<tr>
<td>Early mitral inflow deceleration time</td>
<td>ms</td>
<td>194 ±40 [178-217]</td>
</tr>
<tr>
<td>Mitral early/late velocity ratio</td>
<td>--</td>
<td>1.1 ±0.3 [0.8-1.3]</td>
</tr>
<tr>
<td>Mitral septal annular velocity e′</td>
<td>cm/s</td>
<td>9.3 ±2.9 [7.7-10.9]</td>
</tr>
<tr>
<td>Mitral lateral annular velocity e′</td>
<td>cm/s</td>
<td>12.2 ±3.5 [10.4-13.6]</td>
</tr>
</tbody>
</table>

Table 3. Patient total plasma sulfide and NT-proBNP levels according to diastolic function.

<table>
<thead>
<tr>
<th>Diastolic function</th>
<th>n</th>
<th>Total plasma sulfide (µM)</th>
<th>NT-proBNP (pg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 0</td>
<td>9</td>
<td>1.5 [0.4-2.1]</td>
<td>63 [15-141]</td>
</tr>
<tr>
<td>Grade 1</td>
<td>8</td>
<td>0.8 [0.0-3.3]</td>
<td>106 [19-184]</td>
</tr>
<tr>
<td>Grade 2</td>
<td>7</td>
<td>0.3 [0.0-1.8]*</td>
<td>147 [36-342]*</td>
</tr>
</tbody>
</table>

Data are medians [range]; * p < 0.05, compared to Grade 0 (Wilcoxon rank test)

during diastole. It is often the first sign of ongoing pathological processes in the myocardium. Although the pathological mechanisms behind diastolic dysfunction are not completely understood, it is known to result in the accumulation of various proteins in the extracellular matrix, and to promote fibrosis. (18)

In heart failure, the plasma levels of NT-proBNP are known to rise according to worsening of the clinical signs of pulmonary congestion, NYHA class, and grade of diastolic dysfunction. (19) The echocardiographically defined grade of diastolic dysfunction correlates with invasively measured increased left-ventricular wall stress. (20) Higher plasma NT-proBNP levels correlate with higher ventricular wall tension and higher grade of diastolic dysfunction. (21) NT-proBNP transcription and secretion is activated by left ventricular longitudinal strain. (22) The present study thus confirms significantly higher plasma NT-proBNP levels in patients with Grade 2 diastolic dysfunction, compared to patients with normal diastolic function. (23) This negative correlation was expected according to previous studies with NT-proBNP. (24) and is thus confirmed by our investigations. Hydrogen sulfide is an important gaseous transmitter that modulates vasodilatory effects in the body. (25) It also protects the endothelium through decreased oxidative stress, (26) inhibition of inflammation, (27) and activation of serine phosphorylation of endothelial nitric oxide synthase. (28) All these are well-known mechanisms that promote normal endothelial function. Failure of these mechanisms can lead to endothelial dysfunction, which can cause atherosclerosis and arterial hypertension. Myocardial hypertrophy is a further consequence of endothelial dysfunction. This occurs partly as a reaction to the elevated afterload, and probably partly due to myocardial microcirculation dysfunction, myocytes remodeling, changes in the cellular matrix, and fibrosis. (29) H2S has been shown to intervene in the myocardial fibrosis pathway in hypertensive rats, although the precise mechanism has not been defined yet. (30) As myocardial hypertrophy and fibrosis are hallmarks of hypertrophic heart failure and echocardiographic signs of diastolic dysfunction, we feel that our data fit perfectly into the H2S puzzle. H2S production is down-regulated in hypertrophic and fibrotic myocardium, so we would assume that the lower levels of TPS seen in patients with arterial hypertension and diabetes mellitus represent an...
important sign of the ongoing processes at the cytological level. Although this does not show significant effects on arterial pressure, it does affect the signaling pathways that lead to vascular and myocardial remodeling. (31)

Drachuk et al. (32) showed a direct correlation between low H2S levels and diastolic dysfunction due to aging. As aging is also linked to lower NO production, they demonstrated that supplementation of H2S improved myocardial relaxation and decreased diastolic dysfunction. Therefore, H2S can be used as an independent marker for early diastolic dysfunction in hypertonic patients, which will thus call for immediate action with the relevant approved treatment.

On a more optimistic note, H2S offers a new treatment option. (33) The active metabolite in garlic, allicin, is promptly degraded into organic diallyl polysulfides that are potent H2S donors in the presence of thiols. It has been known for centuries that garlic has substantial medicinal effects and it is considered to be one of the best disease-preventive foods. (34) Garlic reduces the risks associated with cardiovascular disease by lowering of cholesterol level, inhibition of platelet aggregation, and lowering of blood pressure. H2S and its decreased production have been proposed to have an important role in the pathogenesis of arterial hypertension. (35)

Regular aerobic exercise was shown to raise H2S production in the body. Therefore, exercise is also a potential treatment option for vascular remodeling and atherosclerosis, through its provision of H2S donors. (36) Once again, diet and exercise are important in the maintenance of health and the prevention of many diseases, including cardiovascular disease.

As H2S is a gas, this might cause difficulties for its measurement under various clinical conditions. A recent report has also confirmed that H2S is short-lived, and can even be undetectable in normal physiological states. (37) Several different methods have been used to measure H2S and/or sulfide concentrations in biological systems, which have included: head-space gas analysis; derivatization methods, such as entafluorobenzyl bromide or N,N-dimethyl-p-phenylenediamine to form methylene blue; spectrophotometry; a monobromobimane-based assay; and direct measurements in solution with a silver sulfide or polarographic sensor. Due to this wide variety of experimental methods used, highly variable data have been obtained regarding the absolute concentrations of sulfide in the blood and tissues. Thus, there appears to be no general consensus in the field as to which measurement(s) correctly define(s) the ‘biologically available H2S/ sulfide’. (38) In the present study, we used a method to determine the TPS levels (i.e., H2S, dissolved sulfide, acid labile sulfide), to estimate the larger pool of sulfur molecules.

The main factor that might limit the interpretation of our findings here is the low number of patients enrolled, which is partly attributable to the recruitment from a single secondary heart failure clinic, as well as the very often newly diagnosed diabetes mellitus at baseline. Our data thus require confirmation in larger studies. Furthermore, studies are needed to evaluate the prognostic value of serial TPS measurements, and the effects of H2S therapy.

In conclusion, our study has revealed significantly lower TPS levels and higher plasma NT-proBNP levels in patients with diastolic dysfunction Grade 2 compared to patients with normal diastolic function.

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All authors declare no conflict of interest.
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