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Is cystathionine gamma-lyase protein expressed in the heart?

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ABSTRACT

Hydrogen sulfide (H_2S) has emerged as an important gasotransmitter, offering protection against ischemia–reperfusion damage to the heart. Cystathionine gamma-lyase (CSE) is believed to be the major H_2S-generating enzyme in the heart. Quite contrary to the general contemplation, CSE protein in cardiac tissues has not been convincingly detected and it has become an issue of controversy. In the present study, we isolated cardiac tissues from wild type (WT) and CSE knockout mice or the rat. CSE expression at transcriptional and translational levels were assayed by RT-PCR and Western Blotting with five different antibodies (four commercial products and one homemade), respectively. Cardiac H_2S production rate was also examined. Our data validated the expression of CSE mRNA in the heart of WT mice or rats, not in CSE KO mice. Using all 5 different anti-CSE antibodies, we could not detect CSE proteins in mouse or rat cardiac tissues or in cultured rat cardiomyocytes. On the other hand, CSE protein was detectable in liver tissues from WT mice with the expected molecular mass of 43.6 kDa. H_2S production rate of heart tissues in CSE KO mice was significantly decreased compared with that in WT mice. In the presence of an CSE inhibitor, D,L-propargylglycine, H_2S production rate of heart tissues from WT mice was inhibited by approximately 80%. It appears that CSE mediates mostly endogenous H_2S production in heart tissues. However, the available anti-CSE antibodies could not detect CSE proteins in rat and mouse heart tissues or rat cardiomyocytes.

1. Introduction

Cystathionine gamma-lyase (CSE, EC 4.4.1.1) is one of the key enzymes in the cysteine metabolism pathway. It has also been referred as CGL (cystathionine gamma-lyase) or CTH (cystathionase). The mouse CSE open reading frame (ORF) encodes a 43.6 kDa protein with 398 amino acid (aa), which shares high identity with those of the rat (43.6 kDa, 398 aa) and human (44.5 kDa, 405 aa) (93.5% and 85.6%, respectively) [1–3]. CSE is capable of catalyzing the reaction of cystathionine with cysteine to generate hydrogen sulfide (H_2S) [4]. H_2S is an endogenously produced gasotransmitter, which exerts a wide range of actions in our body, especially in cardiovascular system. Administration of H_2S prevented the development of hypertension [5], alleviated hypoxic pulmonary hypertension [6], and limited myocardial infarct size induced by ischemia reperfusion injury [7,8]. The underlying mechanisms for the cardiovascular effects of H_2S involve the inhibition of oxidative stress [9], production of lipid peroxidation and inflammatory factors [10], and activation of ATP sensitive potassium (K_{ATP}) channels [11].

The cardiac protective effect of H_2S has been commonly acknowledged and attributed to the catalytic action of CSE proteins expressed in the heart. As such, the existence of CSE proteins in the heart has been taken for granted. Unfortunately, a careful re-examination of the literature yields a disappointing fact that no solid evidence has been provided for the expression of CSE proteins in cardiac tissues. This fact is sobering as it may lead to some quite different interpretations on many reports, published or to be published, on the role of CSE/H_2S in homeostatic control of cardiac functions. Having aimed at detecting CSE proteins in the heart, we re-evaluated rigorously the expression of CSE gene at both transcriptional and translational levels as well as H_2S production in cardiac tissues. Five different anti-CSE antibodies were used to detect CSE proteins in the hearts from mice and rats as well as in a cloned rat cardiomyocyte cell line. We conclude that although CSE is responsible for the H_2S production in the heart, CSE proteins are undetectable in cardiomyocytes or heart tissues with the antibodies currently available.

2. Materials and methods

2.1. Animals

In-house-bred male (8–10 weeks old, male) CSE knockout and wild type (WT) mice [12] and male Sprague Dawley (SD) rats

Abbreviations: CSE, cystathionine gamma-lyase; PPG, D,L-propargylglycine.

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Table 1
Commercial antibodies applied in this study.

Catalog number	Company	Immunogen	Host	Reactivity	Isotype
C8248[a]	Sigma–Aldrich	Synthetic peptide corresponding to amino acids 22–35 of human CTH	Rabbit	Human	IgG
12217-1-AP[a]	Proteintech Group, Inc.	Residues near the C terminus of human CTH	Rabbit	Human, Mouse, Rat.	IgG
H00001491-M01[a]	Abnova	CTH (AAH15807, 1–405 aa) full-length recombinant protein with GST tag.	Mouse	Guinea pig, Human	IgG_1 Kappa
Sc-100583[a]	Santa Cruz	Recombinant CSE of human origin	Mouse	Mouse and human	IgG

[a] Information cited from manufacturer's instructions.

(10–12 weeks old, male) were purchased from Charles River Laboratories (St.-Constant, QC, Canada). All animal experiments were conducted in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by the Animal Use Committee of Lakehead University (Canada). All animals were housed in a controlled environment with unlimited access to food and water on a 12-h light–dark cycle.

2.2. Cell culture

H9C2 cells (rat cardiomyocyte cell line) were purchased from the American Tissue Type Collection (ATCC, Manassas, VA, USA). Cells were cultured at 37 °C in a humidified incubator with 95% air and 5% CO_2 maintained in Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, and 1.0 mM sodium pyruvate, with 10% fetal bovine serum (FBS) (Invitrogen), 100 units of penicillin, and 100 μg of streptomycin/ml. Cells were grown on 10 cm plates and allowed to become 70–80% confluent.

2.3. Reverse transcription-PCR

Total mRNA was isolated using Trizol (Sigma) and treated with RNase-free DNase (NEB, Beverly, MA) following the manufacturer's instructions. Reverse transcription was performed by using Superscript First Strand synthesis system (Invitrogen). The negative control containing no reverse transcriptase was used to safeguard for genomic DNA contamination in each sample. The primers of CSE (GenBankTM accession number AI314617) were as following: sense 5'-AGCGATTACACCACAAACCAAG-3'; antisense 5'-ATCAGCACCCAGAGCCAAAGG-3'. These primers produced a product of 178 bp [13]. The primers of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are: sense 5'-GTGGAGATTGTTGCACAACG-3'; antisense 5'-CAGTGGATGCAGGGATGATGTTCTG-3' [14].

2.4. Western Blotting and anti-CSE antibodies

For Western Blotting, cultured cells or tissue samples were washed twice in ice-cold PBS, and mixed in a lysis buffer (0.5 M EDTA; 1 M Tris–Cl at pH 7.4; 0.3 M sucrose; 1 μg/ml antipain hydrochloride; 1 mM benzamidine hydrochloride hydrate; 1 μg/ml leupeptin hemisulfate; 1 mM 1,10-phenanthroline monohydrate; 1 μM pepstatin A; 0.1 mM plenylmethylsulfonyl fluoride; and 1 mM iodoacetamide) using a polytron homogenizer. Proteins were loaded at 50 μg/well and separated by standard SDS/PAGE and then transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA) and probed with the following antibodies: anti-CSE (Sigma C8248, 1:1000), anti-CSE (home-made, 1:1000), anti-CSE (Proteintech Group, Inc. 12217-1-AP, Chicago, IL, 1:1000); anti-CTH30.7 (Santa Cruz sc-100583, 1:1000), and anti-CTH (Abnova H00001491-M01, Beverly, MA, 1:1000), respectively. Isotype-matched, horseradish peroxidase-conjugated secondary antibodies (Sigma) were used, followed by detection with chemiluminescence (GE healthcare, Piscataway, NJ). Home-made anti-CSE antibody was prepared as previously described [15]. Briefly, synthesized and conjugated targeted peptide VGLEDEQDLLEDLD (GenBankTM accession number AAL99218.1, position 377-390) was used to immunize male NewZealand rabbits. After the titer of anti-CSE antisera reached 1:4000, the antisera were purified by protein Gaffinity. Purified antibody was eluted with 0.1 M glycine buffer (pH 2.5) and stored at −80 °C for later use. More detailed information about the commercial antibodies is shown in Table 1.

2.5. Measurement of H_2S production rate

H_2S production rate was measured as described previously [2]. Briefly, tissues were homogenized in 50 mM ice-cold potassium phosphate buffer (pH 6.8). The flasks containing the reaction mixture (100 mM potassium phosphate buffer, 10 mM L-cysteine, 2 mM pyridoxal 5-phosphate, and 10% (wt/vol) homogenates) and center wells containing 0.5 ml 1% zinc acetate and a piece of filter paper (2 × 2.5 cm) were flushed with N_2 and incubated at for 90 min. The reaction was stopped by adding 0.5 ml of 50% trichloroacetic acid, and the flasks were incubated at 37 °C for another 60 min. The contents of the center wells were transferred to test tubes, each containing 3.5 ml of water. Then 0.5 ml of 20 mM *N*,*N*-dimethyl-*p*-phenylenediamine sulfate in 7.2 M HCl and 0.5 ml 30 mM $FeCl_3$ in 1.2 M HCl was added. The absorbance of the resulting solution at 670 nm was measured 20 min later with a spectrophotometer.

2.6. Statistical analysis

All data were expressed as mean ± SEM. Statistical analyses between two groups were performed using the unpaired Student's *t*-test. Statistical analysis of more than two groups was performed using one-way ANOVA with Dunnett's multiple comparisons post hoc test. A level of $p < 0.05$ was considered statistically significant.

3. Results

3.1. CSE mRNA expression in heart cells and tissues

We started to identify CSE expression at transcriptional level. RT-PCR results demonstrated that CSE transcripts were detectable in heart tissues from WT mice and SD rats and H9C2 cardiomyocytes. No CSE mRNA could be detected in heart tissues from CSE KO mice. GAPDH was used as a house-keeping gene (Fig. 1).

3.2. H_2S production rate in heart tissues

H_2S produced in heart tissue samples from CSE-KO mice was decreased by approximately 80% compared with the samples from WT mice. Heart tissues were homogenized in 50 mM ice-cold potassium phosphate buffer (pH 6.8) and pretreated with 5 mM PPG (D,L-propargylglycine) for 15 min, an inhibitor of CSE. H_2S production rate in WT cardiac tissues, but not in that from CSE-KO mice, was abolished in the presence of PPG (Fig. 1B).

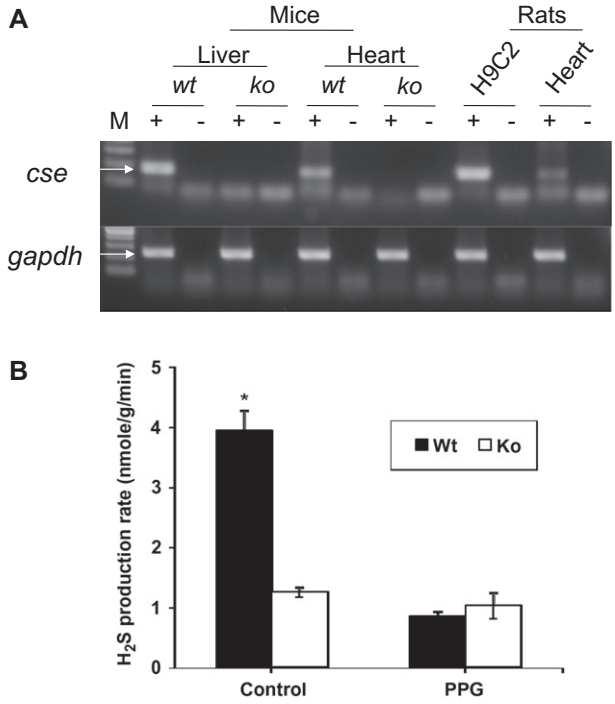

Fig. 1. Detection of CSE transcripts and H_2S production in the heart. (A) RT-PCR analysis of CSE transcripts in liver and heart samples from mice and rats. From left to right: marker (M), samples of livers and hearts from WT and CSE-KO mice, H9C2 cells, and SD rat heart tissues. The arrow in upper panel points to the amplification of *cse* cDNA and the lower panel is *gapdh* cDNA. (−) indicates that the samples are without reverse transcription ($n = 4$). (B) H_2S production from WT and CSE-KO mouse heart tissues with or without PPG treatment ($n = 4$, $p < 0.05$).

3.3. CSE protein expression in heart tissues or cardiomyocytes

To characterize CSE expression at translational level, five antibodies from different sources were used in Western Blotting studies. Among these antibodies, three were polyclonal and the other two were monoclonal. Liver samples from WT and CSE-KO mice were used here as positive and negative control for CSE expression, respectively. The polyclonal antibody from Sigma–Aldrich (Fig. 2A) detected many nonspecific bands in the heart samples from WT mice as well as from CSE-KO mice. No clear band for CSE protein could be identified unambiguously. With our home-made polyclonal antibody (Fig. 2B) and the antibody from Proteintech Group (Fig. 2C), CSE bands were detected in the livers from WT mice, but not in the livers from CSE-KO mice or in any mice heart tissues. With the monoclonal antibodies from Abnova (Fig. 2D) and Santa Cruz (Fig. 2E), CSE protein expression was clearly detected in WT mice livers, not in CSE-KO mice livers or any mice heart samples. These two antibodies also produced non-specific bands in all tested tissues, which would not represent CSE proteins.

CSE protein expression was next examined in rat heart tissues or rat cardiomyocyte line H9C2 cells. Using Sigma's anti-CSE antibody, a 43 kD band was detected in Western Blotting analysis on rat liver, heart tissues, and H9C2 cell lines. The intensity of this band was the weakest with rat heart tissues (Fig. 3A). Our home-made anti-CSE antibody (Fig. 3B), the anti-CSE antibody from Proteintech Group (Fig. 3C), and the Abnova anti-CSE antibody (Fig. 3D) detected a 43 kD CSE band in rat liver, but not in rat heart tissues or H9C2 cells. Sigma's anti-CSE antibody and Abnova's antibody also detected many non-specific bands with different molecular weights (Fig. 3A and D). The Santa Cruz anti-CSE antibody detected one 43 kD band in H9C2 cell sample but it failed to yield any band in rat liver or rat heart tissues (Fig. 3E).

4. Discussion

Endogenously generated H_2S plays important physiological and pathophysiological functions in cardiac protection during ischemia, infarction, and heart failure [7,16–18]. However, whether CSE proteins are expressed in the heart has not been convincingly demonstrated. Some researchers found virtually no CSE proteins in heart tissue samples from mice and/or rats with Western Blotting [1,7], while others detected CSE proteins in heart tissues [19–22]. Ishii et al. [1] described the distribution of CSE proteins in liver, kidney, small intestine and stomach of mice using a home-made rabbit polyclonal anti-CSE antibody. This antibody was constructed against the N terminal amino acid numbers 1–193 of the rat CSE protein. Using the same anti-CSE antibody these authors could not detect CSE proteins in mice hearts (Fig. 5B in [1]). Using the same antibody from Ishii et al. [1], Elrod et al. [7] also could not clearly detect CSE proteins in cardiac tissues from WT mice (Fig. 6C in [7]), but they found *cse* gene expression at mRNA level. After specifically over-expressing CSE (MHC-CGL-Tg) in mouse heart, Elrod et al. [7] were able to show clear Western Blotting bands for CSE in cardiac tissues using the same antibody. It appears that the anti-CSE antibody these authors used can detect CSE proteins but under physiological conditions CSE protein in mouse heart is not detectable.

Zhu et al. [20] identified immunostaining of CSE proteins in the infarct area of the heart from male Wistar rats (250–300 g) with a home-made anti-CSE antibody targeted at an antagonistic piece of rat CSE sequence (Fig. 7 in [20]). The CSE staining was also found in the endothelium of small vessels of area at risk. However, CSE was virtually undetectable in healthy area of the heart. No Western Blotting study was conducted. These authors concluded that "the overexpressed CSE was mainly affected in the injured myocardium". In heart tissues isolated from 6 week-old inbred male Balb/C mice, Western Blotting revealed a positive band with anti-CSE antibody from Proteintech Group (Fig. 2B in [21]). The CSE bands in this study had molecular weight of 45 kD, which is questionable considering the standard molecular weight of mouse CSE protein is about 43.6 kD. Wang et al. (Fig. 5A in [22]) presented the CSE Western Blotting results using an anti-rat CSE polyclonal antibody from R&D Systems. Protein samples were prepared from left ventricular tissue of male Sprague–Dawley rats weighing 250–300 g. The authors concluded that NaHS could stimulate the expression of CSE in left ventricle after myocardial infarction (MI). We had requested R&D Systems for the claimed anti-CSE antibody but, unfortunately, the company stated that it had never carried this product. Qipshidze et al. (Fig. 6A in [19] conducted Western Blotting on heart tissues of male C57BL/6 mice (10 to 14-wk-old). The authors did not mention the provider of the anti-CSE antibody used in the study. A Western Blotting band of 43.5 kD was detected, using this antibody, in mice heart tissues as CSE protein. The same study also detected CBS protein band in mice heart [19].

It is tricky to interpret these published Western Blotting films because most of the reports only showed the "cut-off" film, not the full Western Blotting films with molecular weight markers. The CSE "cut-off bands" did not have clear molecular weight markers associated [7,19,21,22]. Furthermore, without proper controls, including tissues samples in which CSE proteins are known to exist such as liver tissues from wide-type mice in which CSE proteins are known to be absent such as liver tissues from CSE gene knockout mice, a merely detection of a Western Blotting band of approximately 43 kD does not help differentiate non-specific bands from the target CSE proteins.

We examined CSE expression in cardiac tissues at transcriptional, translational and enzymatic activity levels in the present

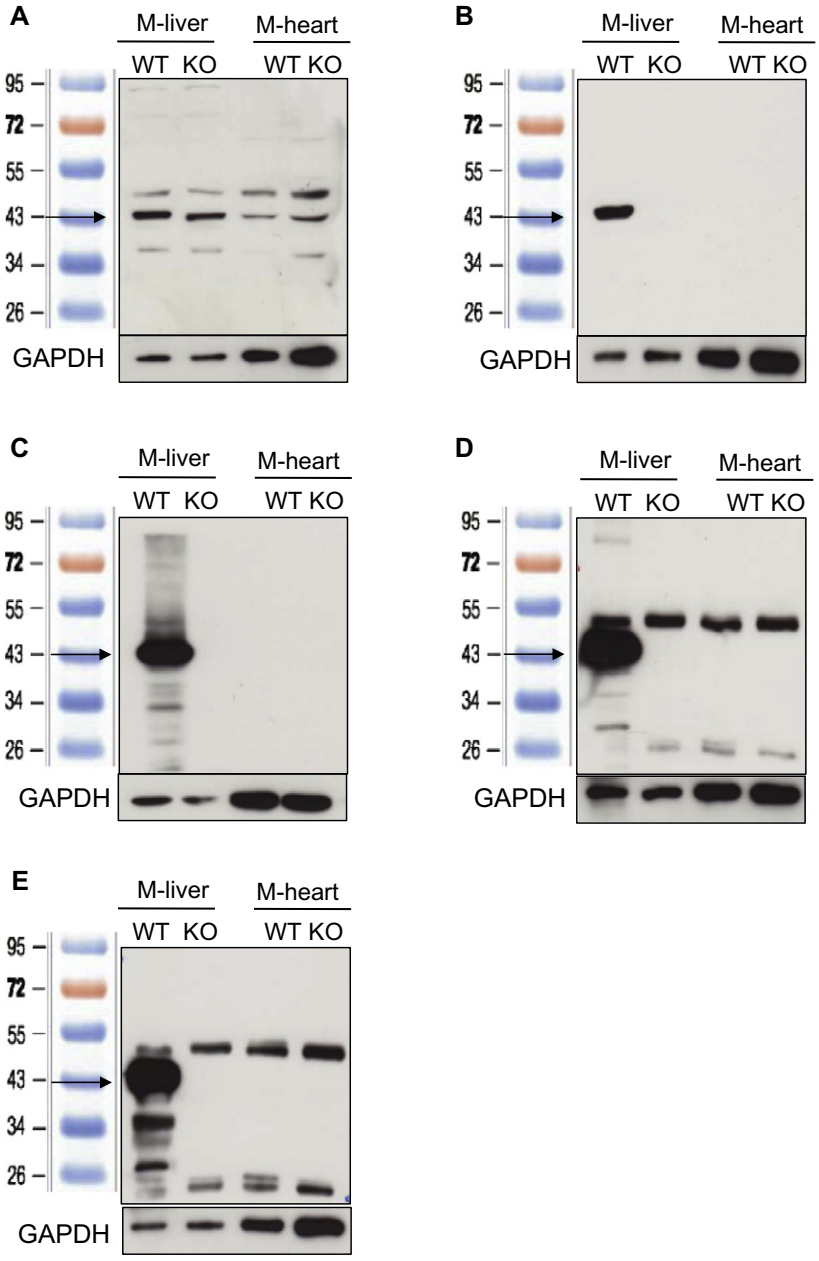

Fig. 2. Western Blotting detection of CSE proteins in mouse heart tissues. The arrow indicates the target CSE molecular weight ($n = 4$). (A) Anti-CSE antibody from Sigma. (B) Home-made anti-CSE antibody. (C) Anti-CSE antibody from Proteintech Group. (D) Anti-CSE antibody from ABNOVA. (E) Anti-CSE antibody from Santa Cruz.

study. In cardiac tissues of WT mice and rats' cardiomyocytes CSE transcripts were clearly detected. H_2S production was also recorded in WT mouse cardiac tissues, which was significantly reduced in the presence of PPG or largely eliminated in CSE-KO mouse heart (Fig. 1B). These results indicate the presence of CSE gene and its enzymatic activity in the heart [7,16,20,21]. However, using different anti-CSE antibodies, we cannot confirm the existence of CSE proteins in the hearts of mice or rats (Figs. 2 and 3). CSE mRNA and proteins are absent in any tissues from CSE-KO mice. Tissues from CSE-KO mice serve as the negative control to assure the specificity of the anti-CSE antibodies used in Western Blotting assays. In our study, most of the anti-CSE antibodies used has selectivity and affinity toward CSE proteins in WT mouse and rat liver tissues. Once CSE gene is knockout, these antibodies do not reveal any positive Western Blotting bands in liver tissues of CSE-KO mice (Figs. 2B–E and 3B–D). The Santa Cruz's anti-CSE antibody is one exception that could not detect CSE proteins in rat liver samples (Fig. 3E). Another exception is Sigma's anti-CSE antibody (Fig. 2A) that detected the positive bands with the same molecular weights of 43 kD in liver tissues from both WT and CSE-KO samples. This antibody also detects multiple non-specific bands in liver and heart tissues from mice or rats. The selectivity and specificity of Sigma's anti-CSE antibody are questionable. Although this antibody detected a 43 kD band in rat heart tissues (Fig. 3A), given that the same consideration aforementioned, it is difficult to associate the 43 kD bands detected with this Sigma's anti-CSE antibody to the identity of CSE proteins in mice or rats, no matter liver or heart or other tissues.

Moreover, the anti-CSE antibodies from Abnova and Santa Cruz yielded pseudo positive bands in liver and heart tissues from both

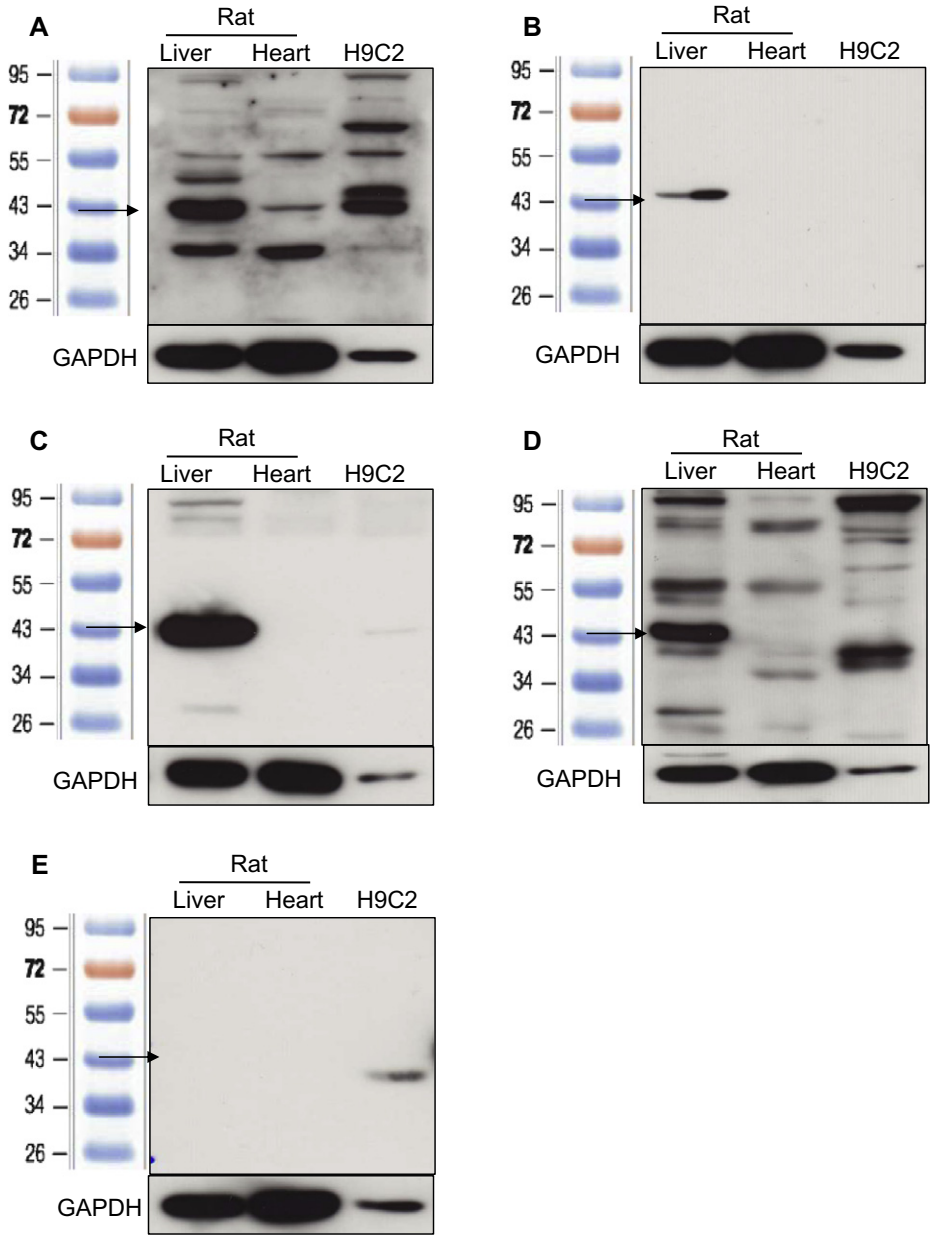

Fig. 3. Western Blotting detection of CSE proteins in rat tissues and H9C2 cells. The arrow points to the bands of 43 kD which may represent CSE proteins ($n = 4$). (A) Anti-CSE antibody from Sigma. (B) Home-made anti-CSE antibody. (C) Anti-CSE antibody from Proteintech Group. (D) Anti-CSE antibody from ABNOVA. (E) Anti-CSE antibody from Santa Cruz.

WT and CSE KO mice with molecular weights significantly greater than 43 kD (Fig. 2D and E). These bands are unlikely the indication of CSE proteins. It may argue that one can sequence the questioned positive bands in Western Blotting gels to assure whether a specific protein is targeted. However, numerous proteins, related or not related to CSE, may be present at the same location on the gel. To sequence all proteins in one location of Western Blotting gel is nearly impossible.

The failure of detecting CSE proteins in the heart in our study is not due to protein degradation. We have included seven different proteases inhibitors (antipain hydrochloride, benzamidine hydrochloride hydrate, leupeptin hemisulfate, 1,10-phenanthroline monohydrate, pepstatin A, plenylmethylsulfonyl fluoride and iodoacetamide) in the lysis buffer for tissues lysates preparation to prevent from protein degradation. The same lysis buffer has been used for liver tissues and CSE proteins are clearly detected in livers.

We conclude that in rat and mouse hearts CSE mRNA is expressed and H_2S is endogenously produced. However, the available anti-CSE antibodies could not detect CSE proteins in rat and mouse heart tissues or rat cardiomyocytes. Does CSE protein exist in cardiac tissue? Indirect lines of evidence would cast a yes vote on this. H_2S generation in cardiac tissues is significantly decreased not only after inhibiting CSE with PPG in WT mice but also in CSE-KO mice. The turnaround time of CSE protein in the heart has not been examined. CSE proteins might be degraded faster in the heart so that its mRNA can, but CSE proteins cannot, be detected. CSE heterogeneity was reported in human. Two isoforms of CSE were detected in human liver [23]. However, *cse* gene in the heart has not been cloned or sequenced. The existence of different isoforms of CSE proteins in the heart may be another explanation on the failure of the currently available anti-CSE antibodies to detect CSE proteins in the heart.

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