Hyperhomocysteinemia and Myocardial Expression of Brain Natriuretic Peptide in Rats

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Background: Hyperhomocysteinemia (HHcy) has been linked to impaired left ventricular function and clinical class in patients with chronic heart failure. We hypothesized that HHcy stimulates myocardial brain natriuretic peptide (BNP) expression and induces adverse left ventricular remodeling.

Methods: We randomized 50 rats into 5 groups. Groups Co1 and Co2 (controls) received a typical diet. Groups Meth, Hcy1, and Hcy2 were fed the same diet supplemented with 2.4% methionine, 1% homocystine, and 2% homocystine, respectively. After 12 weeks, we measured total plasma homocysteine (tHcy) and BNP in plasma and tissue, and we performed histomorphometric analyses.

Results: All animals had comparable baseline body weight [mean (SD) 234 (26) g] and total circulating Hcy [4.7 (1.7) μmol/L]. After 12 weeks of treatment, total circulating Hcy increased in Meth, Hcy1, and Hcy2 [27.3 (8.8), 40.6 (7.0), and 54.0 (46.0) μmol/L, respectively] and remained unchanged in Co1 and Co2. Serum BNP significantly increased in 1 of 10 animals in Meth, 3 of 10 animals in Hcy1, and 3 of 10 animals in Hcy2. Median [25th–75th percentile] BNP tissue concentrations in Hcy1 and Hcy2 were 55% higher than in the corresponding controls [Co1 vs Hcy1, 225 (186–263) vs 338 (262–410) pg/mg protein, P = 0.05; Co2 vs Hcy2, 179 (107–261) vs 308 (192–429) pg/mg protein, P = 0.12]. In the Meth group, BNP expression was comparable to that of controls [200 (159–235) vs 225 (186–263) pg/mg protein, P = 0.32]. The percentage of perivascular and interstitial collagen and mast cell infiltration were comparable in all groups, indicating no adverse cardiac remodeling.

Conclusion: Three months of intermediate HHcy stimulated increased cardiac BNP expression that was not accompanied by adverse cardiac remodeling.

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Hyperhomocysteinemia (HHcy) and chronic heart failure (CHF) are frequent problems of elderly individuals and often coincide (1). Recent epidemiologic studies from Europe and the US consistently found that the frequency of HHcy was >25% in persons ≥55 years old (2) and increased continuously with age. CHF affects 5 million patients in the US, with ~550 000 newly diagnosed cases each year (3). Hospital discharges for CHF increased from 399 000 in 1979 to 1 093 000 in 2003, and the 2003 overall death rate for CHF was 19.7% (3). A similar situation exists in Europe (4). Consequently, prevention of CHF by identifying and modifying risk factors is a major issue.

Previous analyses found hypertension, smoking, diabetes mellitus, obesity, and advancing age to be the most important risk factors for CHF (5). Recent clinical and experimental studies suggest that HHcy is also a risk factor for CHF (1, 6, 7). The prevalence of HHcy in CHF patients is 50%–60%, depending on the cutoff applied (8, 9). Results from the prospective Framingham Heart Study demonstrated an almost doubled incidence of CHF in individuals with a total circulating Hcy (tHcy) above

Nonstandard abbreviations: HHcy, hyperhomocysteinemia; CHF, chronic heart failure; Hcy, homocysteine; tHcy, total circulating homocysteine; NT-proBNP, N-terminal pro-BNP; BNP, brain natriuretic peptide; Co1, Co2, control group 1 and 2; Meth, methionine group; Hcy1, homocystine 1% group; Hcy2, homocystine 2% group.
the sex-specific median (1). In previous studies from our group, analyzing >1000 individuals, we found a consistent relation of tHcy with the severity of CHF (6, 9). The closest relation could be observed between tHcy and N-terminal pro–brain natriuretic peptide (NT-proBNP). A recent study from Korea confirmed this finding (10). In animal experiments from the group of Joseph and Kennedy (11, 12), chronic intermediate HHcy induced cardiac hypertrophy, pathological remodeling (11, 13, 14), and diastolic as well as systolic dysfunction (11, 12). These findings provide strong evidence for a causal role of HHcy in the pathogenesis of left ventricular dysfunction.

Brain natriuretic peptide (BNP) and NT-proBNP are laboratory markers of CHF with a high negative predictive value (15–17). They are increased in systolic and diastolic dysfunction (18, 19) and have been found to be useful for differential diagnosis of dyspnea in CHF and pulmonary diseases (20). Especially in early disease stages, NT-proBNP seems to be more sensitive than echocardiography (21, 22). Moreover, measurements of BNP and NT-proBNP are free from subjective influences and have good reproducibility (23). In this study, we investigated myocardial BNP expression and cardiac remodeling in healthy adult rats after 3 months of moderate or intermediate HHcy.

Materials and Methods

ANIMALS AND STUDY DESIGN

After 7 days of acclimatization, 50 10- to 12-week-old female Wistar rats (180 to 292 g; Charles River GmbH) were randomized into 5 groups of 10 animals each: 2 control groups (Co1 and Co2) and 3 groups with HHcy. Animals were maintained on a 12 h:12 h light-dark cycle in our institutional stable with free access to food and water. HHcy was induced by 3 different dietary regimens. The first HHcy group (Meth) received a diet that was enriched with 24 g methionine (Sigma) per kg food (corresponds to 2.4% methionine). The 2 other groups were fed with homocysteine-enriched diets, containing 10 g (Hcy1) or 20 g (Hcy2) homocystine (Sigma) per kg food (corresponding to 1% and 2% Hcy). Except for the added methionine and homocystine, all diets were of identical composition. Diets were purchased from Aliments; 0.2 g/L KCl, 0.2 g/L KH2PO4, 8.0 g/L NaCl, 1.15 g/L Na2HPO4, and we measured the total protein concentration using a Bradford assay (Bio-Rad). We quantified BNP with the same method that was previously described for the quantification in plasma. The recovery rate from tissue is 85% with this assay. The stability of these aliquots was used for the quantification of tissue BNP concentration.

BLOOD CHARACTERISTICS

We used heparin plasma samples to measure tHcy, BNP, folate, vitamin B12, and creatinine. tHcy was analyzed with an enzymatic fluorescence polarization immunoassay on an Axsym analyzer (Abbott). Intra- and interassay CVs of this method were 4.5% and 5.1%, respectively. We quantified BNP by use of the rat BNP-45 ELISA reagent set (Gentaur), using a polyclonal antibody specific for BNP-45. BNP-45 represents the circulating (active) form of BNP in rats and is equivalent to BNP-32 in humans. Intra- and interassay CVs of the BNP ELISA were 5.3% and 8.5% at concentrations of 100 ng/L. For this assay, the recovery rate from plasma is 89%, and the reference range of healthy untreated rats is below the detection limit of 10 ng/L. Folate and vitamin B12 were detected with chemiluminescence immunoassays on an ACS Centaur analyzer (Bayer Healthcare). Intra- and interassay CVs were 4.0% and 4.4% at a concentration of 208 ng/L for vitamin B12, and 5.3% and 5.5% at a concentration of 5.3 μg/L for folate. We measured creatinine by use of a colorimetric assay using the alkaline picate method on a Hitachi 917 analyzer (Roche Diagnostics).

BNP TISSUE CONCENTRATION

One deep frozen tissue aliquot per ventricle was thawed and homogenized with a mortar. The homogenized tissue was dissolved in phosphate buffered saline (PAA Laboratories; 0.2 g/L KCl, 0.2 g/L KH2PO4, 8.0 g/L NaCl, 1.15 g/L Na2HPO4), and we measured the total protein concentration using a Bradford assay (Bio-Rad). We quantified BNP with the same method that was previously described for the quantification in plasma. The recovery rate from tissue is 85% with this assay. The stability of tissue BNP-45 at −20 °C is 3 months. Because tissue aliquots had different volumes and masses, BNP values were expressed relative to the protein content of each aliquot.

HISTOMORPHOMETRY

We performed morphometric analyses as previously described by Southern et al. (24). After dehydration with increasing alcohol concentrations, aliquots were embed-
duced in paraffin, and 5-µm sections from 2 locations 100 µm apart were prepared. After removal of the paraffin, sections were stained with picrosirius red (Sigma), which is specific for fibrillar collagen (25).

To avoid subjective influences on morphometric results, sections were blinded before analysis. We prepared digital images from 15 microscopic fields per section and analyzed 2 sections per ventricle, 1 per location. The interstitial collagen fraction was calculated as the percentage of red-colored interstitial regions relative to the total area. For the quantification of perivascular collagen, we analyzed 5 vessels per section with a diameter of 50–200 µm and expressed perivascular collagen relative to the vessel luminal area. Serial measurements of the same section revealed a variation of 1% for the analysis of perivascular and interstitial collagen.

Previous work by Joseph et al. showed a protective role of mast cells in Hcy-induced cardiac remodeling (26). Therefore, we also analyzed myocardial mast cell infiltration using toluidine blue staining (27). Sections from both locations were rehydrated and incubated for 30 min in 0.001% toluidine blue solution (Sigma). In 2 sections per ventricle, 1 per location, mast cells (blue) were counted and expressed relative to the analyzed area.

BNP EXPRESSION IN EX VIVO SUPERFUSED RAT MYOCARDIUM

To confirm the BNP results in vivo, we performed superfusion experiments with freshly prepared rat myocardium from three 10- to 12-week-old Wistar rats (mean weight 260 g; Charles River) as described previously by Jeron et al. (28). Briefly, after explantation of the heart and isolation of the left ventricle, we prepared tissue slices (0.35 mm thick) from the left ventricle (48 slices per heart) and transferred them immediately to minisuperfusion chambers (volume 80 µL). We performed superfusion for 13 h at a temperature of 37 °C and a flow rate of 66 µL/min (1 slice per chamber, 48 chambers in parallel) with culture medium containing RPMI 1640 (Sigma), 25 mmol/L HEPES (Roth), 5% fetal calf serum (PAA Laboratories), 30 µmol/L mercaptoethanol (Sigma), 0.57 mmol/L ascorbic acid (Merck), 1.3 mmol/L calcium (Merck), 100 000 IU/L penicillin, and 100 mg/L streptomycin (Invitrogen Gibco).

During the first 2 h of superfusion, we superfused tissue slices with culture medium without further stimulation (wash-in period). From 2–13 h, Hcy-conditioned medium was applied continuously at concentrations of 0, 10, 50, and 200 µmol/L to modulate BNP secretion. Each Hcy concentration was applied to 8 tissue slices per rat (8 slices in 3 rats = 24 total).

We collected superfuse aliquots for 15 min at 2, 3, 5, 7, 9, 11, and 13 h and immediately froze them at −80 °C until measurements. We quantified BNP with the same rat BNP-45 ELISA reagent set (Gentaur) as used for serum and tissue samples. After the end of the superfusion period, tissue slices were homogenized and protein concentration was measured using the BCA-Protein assay (Pierce). BNP secretion was expressed as picograms per microgram protein.

STATISTICS

Based on results of a Kolmogorov–Smirnov test, all variables, except of tissue BNP, showed gaussian distribution. Results of the descriptive statistics are expressed as mean (SD). Group comparisons of gaussian-distributed variables were performed using 1-way ANOVA with least significant difference post hoc test. Tissue BNP, which was not gaussian distributed, was compared using a Kruskal–Wallis or a Mann–Whitney test. All calculations were performed using the SPSS 11.0 software package (SPSS Inc.).

Results

ANIMAL MODEL

At the beginning of the study, animals had comparable baseline characteristics. Mean (SD) body weight was 234 (26) g and tHcy was 4.7 (1.7) µmol/L. After 12 weeks of treatment, Meth, Hcy1, and Hcy2 animals exhibited significant HHcy (Table 1). The Meth group developed moderate HHcy, and the Hcy1 and Hcy2 groups had intermediate HHcy, with the highest Hcy concentrations.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Co1</th>
<th>Meth</th>
<th>Hcy1</th>
<th>Co2</th>
<th>Hcy2</th>
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<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>306 (20)</td>
<td>259 (17)*</td>
<td>322 (17)</td>
<td>345 (28)</td>
<td>254 (28)*</td>
</tr>
<tr>
<td>Change in body weight, g</td>
<td>63 (19)</td>
<td>53 (15)</td>
<td>85 (14)</td>
<td>100 (28)</td>
<td>8.5 (25)*</td>
</tr>
<tr>
<td>Hcy, µmol/L</td>
<td>4.6 (1.5)</td>
<td>27.3 (8.8)*</td>
<td>40.6 (7.0)*</td>
<td>7.0 (1.6)</td>
<td>54.0 (46.0)*</td>
</tr>
<tr>
<td>Folic acid, µg/L</td>
<td>53 (10)</td>
<td>56 (9)</td>
<td>55 (4)</td>
<td>46 (16)</td>
<td>64 (13)*</td>
</tr>
<tr>
<td>Vitamin B12, ng/L</td>
<td>1180 (174)</td>
<td>1132 (248)</td>
<td>1016 (158)</td>
<td>1392 (157)</td>
<td>1177 (353)</td>
</tr>
<tr>
<td>Creatinine, µmol/L</td>
<td>45.1 (9.7)</td>
<td>56.6 (13.3)</td>
<td>48.6 (11.5)</td>
<td>47.7 (2.7)</td>
<td>36.2 (5.3)*</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>405 (26)</td>
<td>401 (43)</td>
<td>393 (27)</td>
<td>391 (23)</td>
<td>413 (32)</td>
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<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>136 (12)</td>
<td>131 (10)</td>
<td>142 (10)</td>
<td>144 (8)</td>
<td>124 (8)</td>
</tr>
</tbody>
</table>

* Data are mean (SD). Three of 10 animals from group Co2 had to be excluded because of spontaneously increased Hcy levels >10 µmol/L. Two of 10 animals from group Hcy2 had to be excluded because Hcy concentrations at the end of the treatment were <10 µmol/L.

a P < 0.001 vs Co1; * P < 0.001 vs Co2; ** P < 0.05 vs Co2.
in the Hcy2 group. We excluded 3 animals from the Co2 group because they exhibited spontaneously increased Hcy concentrations >10 μmol/L. We excluded 2 animals from the Hcy2 group because their Hcy concentration at the end of the treatment was <10 μmol/L.

Plasma creatinine was within reference intervals in all animals [50.4 (9.7) μmol/L]. Moreover, Meth, Hcy1, and Hcy2 groups did not show higher plasma creatinine than controls (Table 1). All animals exhibited high plasma concentrations of folate and vitamin B12 that did not differ between groups (Table 1). Body weight was significantly lower in Meth and Hcy2 compared with controls, owing to reduced food intake in these groups. Heart rate and blood pressure did not differ between groups.

### BNP Expression

At the end of the study, plasma BNP was below the detection limit of 10 ng/L in all controls. One Meth animal, 3 Hcy1 animals, and 3 Hcy2 animals showed an increase in circulating BNP (Fig. 1). Because many of the animals in the HHcy groups exhibited undetectable BNP concentrations at the end of the study, the calculation of mean values was not meaningful. Therefore, we categorized animals as BNP positive and negative. The number of BNP-positive animals increased with higher circulating Hcy concentrations (Fig. 1). Introducing categorized BNP values in a cross table, Pearson χ² test revealed a significant relation between circulating Hcy and plasma BNP (χ² = 7.89, P = 0.048). Pooling all controls and all HHcy animals in 2 groups, we confirmed a higher number of BNP-positive animals in the HHcy group (χ² = 5.03, P = 0.025).

Because most of the animals had undetectable BNP plasma concentrations, we quantified tissue concentrations of BNP in the myocardium (Fig. 2). For animals in the Hcy1 and Hcy2 groups, BNP tissue concentrations were ~55% higher than in controls. In the Hcy1 group, this difference was significant (P = 0.05). Group comparison between Co2 and Hcy2 revealed only a trend (P = 0.12) because of larger interindividual variation of BNP tissue concentrations in the Hcy2 group.

To confirm the results described above and to demonstrate causality between circulating Hcy and BNP expression, we performed superfusion experiments stimulating freshly prepared rat myocardium slices with increasing concentrations of Hcy. The mean BNP secretion during the 11 h of Hcy superfusion increased significantly with higher Hcy concentrations in the superfusion medium (Fig. 3).

### Left Ventricular Remodeling

The relative weight of the left ventricle in Meth and Hcy1 groups was comparable to the corresponding controls. Only Hcy2 showed a significant increase, by 15% (Fig. 4A). Similarly, lung weight, as a measure of interstitial fluid, did not differ from controls in the Meth and Hcy1 groups (Fig. 4B). Only the Hcy2 group exhibited a significantly higher lung weight, indicating congestion.

Because ventricular and lung weights are relatively raw measures, we performed morphometric analyses of interstitial and perivascular collagen. Interstitial collagen was 2.8%–5.1% and was not different between groups (Fig. 5A). Perivascular collagen was comparable in Co1, Co2, Meth, and Hcy1 groups, at 2.0%–2.1% (Fig. 5B). The Hcy2 group exhibited a perivascular collagen of 2.7%,
The main finding of the present study was a significant induction of cardiac BNP expression by chronically increased tHcy concentrations. Higher BNP concentrations were not accompanied by an adverse remodeling of the left ventricle, the main source of BNP. The causality between HHcy and increased BNP expression was demonstrated by ex vivo superfused left ventricular tissue slices, which were stimulated with various Hcy concentrations. HHcy was induced by 3 different models. According to current concepts, in humans HHcy can be classified as moderate (12–30 μmol/L), intermediate (30–100 μmol/L), or severe (>100 μmol/L) (29, 30). Based on this classification, moderate HHcy was induced in the Meth group by the addition of 2.4% methionine to the typical diet. A comparable model of moderate HHcy has been described by Woo et al. (31). The mean tHcy in Meth was comparable with the findings of Woo et al. (27.3 vs 25.3 μmol/L). Intermediate HHcy was created with a homocystine-enriched diet (1%), as published by Joseph et al. (12, 14). tHcy was similar to tHcy values reported by Joseph et al. (40.6 vs 32.7 μmol/L (12) and vs 47.1 μmol/L (14)). To further increase (Hcy, we augmented the homocystine concentration (2%) in the diet of the 3rd group. This diet caused a mean (Hcy of 54.0 μmol/L; however, animals of the Hcy2 group exhibited large interindividual variations in circulating Hcy, and 3 animals had to be excluded because they did not show increased Hcy concentrations. Compared with the corresponding controls (Co2), weight gain was significantly lower, indicating a reduced tolerance to this diet and suggesting that a homocystine concentration of 2% is probably the maximum that can be administered.

This study demonstrates a significant stimulation of BNP expression, a sensitive marker of myocardial dysfunction and increased wall stress (21, 22), with a high negative predictive value (15–17) by intermediate HHcy (Hcy1 and Hcy2), but not by moderate HHcy (Meth). This

Discussion

The main finding of the present study was a significant induction of cardiac BNP expression by chronically increased tHcy concentrations. Higher BNP concentrations were not accompanied by an adverse remodeling of the left ventricle, the main source of BNP. The causality between HHcy and increased BNP expression was demonstrated by ex vivo superfused left ventricular tissue slices, which were stimulated with various Hcy concentrations. HHcy was induced by 3 different models. According to current concepts, in humans HHcy can be classified as moderate (12–30 μmol/L), intermediate (30–100 μmol/L), or severe (>100 μmol/L) (29, 30). Based on this classification, moderate HHcy was induced in the Meth group by the addition of 2.4% methionine to the typical diet. A comparable model of moderate HHcy has been described by Woo et al. (31). The mean tHcy in Meth was comparable with the findings of Woo et al. (27.3 vs 25.3 μmol/L). Intermediate HHcy was created with a homocystine-enriched diet (1%), as published by Joseph et al. (12, 14). tHcy was similar to tHcy values reported by Joseph et al. (40.6 vs 32.7 μmol/L (12) and vs 47.1 μmol/L (14)). To further increase (Hcy, we augmented the homocystine concentration (2%) in the diet of the 3rd group. This diet caused a mean (Hcy of 54.0 μmol/L; however, animals of the Hcy2 group exhibited large interindividual variations in circulating Hcy, and 3 animals had to be excluded because they did not show increased Hcy concentrations. Compared with the corresponding controls (Co2), weight gain was significantly lower, indicating a reduced tolerance to this diet and suggesting that a homocystine concentration of 2% is probably the maximum that can be administered.

This study demonstrates a significant stimulation of BNP expression, a sensitive marker of myocardial dysfunction and increased wall stress (21, 22), with a high negative predictive value (15–17) by intermediate HHcy (Hcy1 and Hcy2), but not by moderate HHcy (Meth). This

![Fig. 3. Mean BNP secretion (95% confidence interval (CI)) of freshly prepared rat myocardium slices during 13 h of superfusion with Hcy-enriched RPMI 1640 medium. The experiment was performed with 3 animals and 8 slices in each animal. For each Hcy concentration, 24 tissue slices (8 per animal) were analyzed. The P value above the horizontal line represents the result of the 1-way ANOVA including all experimental groups. **P < 0.01 vs 0 μmol/L Hcy (least significant difference post hoc test).](image)

![Fig. 4. Relative heart (A) and lung (B) weights after 12 weeks of feeding. Co1, control group 1st run; Co2, control group 2nd run; Meth, methionine group; Hcy1, homocystine 1% group; Hcy2, homocystine 2% group. **P < 0.01 vs Co1; ***P < 0.01 vs Co2.](image)
BNP-inducing effect was demonstrated in plasma as well as in myocardial tissue and confirms the results of 2 previous studies, in which we demonstrated a significant positive relation between Hcy and NT-proBNP in CHF patients (6, 9). The changes in BNP were not accompanied by adverse cardiac remodeling, as indicated by lack of change in interstitial and perivascular collagen in all groups. Moreover, the number of mast cells did not differ between controls and HHcy groups. The discrepancy between BNP expression and cardiac remodeling is not surprising, since BNP probably represents a more sensitive indicator to detect cardiac changes. Increases of BNP and NT-proBNP can frequently be found in diastolic dysfunction (18, 19, 32) in which the ejection fraction is normal, as well as in mild systolic dysfunction (21, 22). In addition, modern concepts consider the heart to be a multifunctional and interactive organ that is part of a complex network (33, 34). Several studies have shown that BNP has antifibrotic and cytoprotective properties (35, 36). Obviously, these counterregulatory actions of BNP are present before clinical symptoms and before adverse cardiac remodeling.

Contrary to histomorphometry, BNP assessment is also free from subjective modification. The sensitivity of currently available rat BNP assays is limited, however. The ELISA used in the current study could not quantify plasma BNP in control animals (all control animals had undetectable plasma BNP concentrations). Consequently, a reference interval could not be defined. Because of the limited sensitivity of the rat BNP assay, we could not see potential changes of plasma BNP below the detection limit. Therefore, the measurement of tissue BNP has to be considered more reliable, since all animals exhibited concentrations above the detection limit and in the linear range of the assay. Hcy1 and Hcy2 groups showed a 55% increase of BNP tissue expression, which supports the result in plasma. Unchanged BNP tissue concentrations were observed in the Meth group. The causality between Hcy and BNP expression was demonstrated by the ex vivo superfusion of freshly prepared rat myocardial slices with Hcy-enriched medium. Hcy concentrations of 50 and 200 μmol/L caused a significant increase of BNP secretion by 60% and 140%, respectively.

What is the explanation for the absence of BNP induction in the Meth group? An obvious reason could be the lower tHcy in these animals. However, even if tHcy was significantly lower than in the Hcy1 and Hcy2 animals, it was clearly above control concentrations. At baseline, all controls had tHcy <10 μmol/L. Therefore, a mean tHcy of 27.3 μmol/L corresponds to at least triple normal values. A more probable explanation is that methionine and homocystine caused HHcy by different biochemical mechanisms with different consequences. Homocystine is the disulfide of 2 Hcy molecules. Addition of homocystine to the diet probably increases tHcy directly and leads to a product excess in methionine metabolism, inhibiting transmethylation and thereby the utilization of methionine. In contrast, a high intake of methionine induces a substrate excess in methionine metabolism that is probably accompanied by increased transmethylation activity. Because hypomethylation is a major mechanism of adverse Hcy effects (37, 38), this biochemical difference between the diets could explain the absent BNP induction in the Meth group. However, this explanation is still not proven.

Contrary to previous work by the group of Joseph et al. (11–14, 26), we could not find any morphologic evidence for adverse cardiac remodeling. This result was surprising, because we used exactly the same dietary model and the same methods to detect cardiac remodeling. The increase of perivascular and interstitial collagen observed by Joseph et al. was 50%–100% in normotensive rats (12) and 100%–500% in hypertensive rats (14). Both studies included fewer than 10 animals per group. Our investigation comprised 28 HHCy animals and 17 controls. These relatively large numbers lend our study a strong impact.
At present, we cannot find any explanation for the discrepancy between the results by Joseph et al. and ours. Because Joseph et al. confirmed their findings in several studies (11–14, 26), there is no reason to doubt them. However, our experiments were performed in 2 independent runs, and all variables analyzed consistently suggested no relevant cardiac remodeling after 12 weeks of HFrE. These conflicting findings must be clarified by further studies.

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