Published in final edited form as:

Mol Genet Metab. 2011 August ; 103(4): 330–337. doi:10.1016/j.ymgme.2011.04.012.

Constitutive induction of pro-inflammatory and chemotactic cytokines in cystathionine beta-synthase deficient homocystinuria

Amy K. Keating^a, Cynthia Freehauf^a, Hua Jiang^a, Gary L. Brodsky^a, Sally P. Stabler^b, Robert H. Allen^b, Douglas K. Graham^a, Janet A. Thomas^a, Johan L.K. Van Hove^a, and Kenneth N. Maclean^{a,*}

^aDepartment of Pediatrics, University of Colorado School of Medicine, Aurora, CO, USA

^bDepartment of Medicine, University of Colorado School of Medicine, Aurora, CO, USA

Abstract

Cystathionine beta-synthase (CBS) deficient homocystinuria (HCU) is an inherited metabolic defect that if untreated, typically results in cognitive impairment, connective tissue disturbances, atherosclerosis and thromboembolic disease. In recent years, chronic inappropriate expression of the inflammatory response has emerged as a major driving force of both thrombosis and atherosclerotic lesion development. We report here a characterization of the abnormalities in cytokine expression induced in both a mouse model of HCU and human subjects with the disease in the presence and absence of homocysteine lowering therapy. HCU mice exhibited highly significant induction of the pro-inflammatory cytokines IL-1alpha, IL-1beta and TNF-alpha. Similarly, in untreated/poorly compliant human subjects with HCU we observed constitutive induction of multiple pro-inflammatory cytokines (IL-1alpha, IL-6, TNF-alpha, IL-17 and IL-12(p70)) and chemotactic chemokines (fractalkine, MIP-1alpha and MIP-1beta) compared to normal controls. These HCU patients also exhibited significant induction of IL-9, TGF-alpha and G-CSF. The expression levels of anti-inflammatory cytokines were unaffected in both HCU mice and human subjects with the disease. In the human subjects, homocysteine lowering therapy was associated with either normalization or significant reduction of all of the pro-inflammatory cytokines and chemokines investigated. We conclude that HCU is a disease of chronic inflammation and that aberrant cytokine expression has the potential to contribute to multiple aspects of pathogenesis. Our findings indicate that anti-inflammatory strategies could serve as a useful adjuvant therapy for this disease.

Keywords

Cystathionine beta-synthase; Cytokine; Homocystinuria; Homocysteine; Interleukin-1; Tumor necrosis factor alpha

1. Introduction

Cystathionine β -synthase (CBS) (EC 4.2.1.22, CBS) represents a key regulatory point at the first committed step in the biosynthesis of cysteine via the transsulfuration pathway. The CBS enzyme catalyzes a pyridoxal 5'-phosphate (PLP)-dependent β -replacement reaction condensing serine and homocysteine (Hcy) to form cystathionine which is subsequently converted to cysteine by the action of cystathionine γ -lyase (CGL) [1]. CBS deficiency is the most common cause of homocystinuria (HCU) which, if untreated, causes mental retardation, a range of connective tissue disturbances including Marfanoid skeletal abnormalities, osteoporosis, lens dislocation and a dramatically increased incidence of vascular disorders including atherosclerosis and thromboembolic disease [1]. Moderately elevated plasma total Hcy (tHcy) in humans has been identified as a major risk factor for cardiovascular and thromboembolic disease [2,3]. Although recent data has cast some doubt regarding the causative nature between mild elevations in tHcy and cardiovascular disease (CVD) [4,5] there is little ambiguity regarding the connection between the metabolic changes induced by HCU and thrombosis. One previous analysis estimated that an untreated patient with pyridoxine non-responsive HCU has a 27% chance of having a thrombotic event by the age of 15 [6]. Cardiovascular complications remain the primary cause of morbidity in this disease [1].

Treatment for pyridoxine non-responsive HCU currently consists of a combination of dietary supplementation with betaine and restricted dietary intake of the Hcy precursor methionine. Betaine lowers tHcy levels by serving as a methyl donor in the remethylation of Hcy to methionine catalyzed by betaine-homocysteine S-methyltransferase [1]. Although the incidence of CVD in pyridoxine non-responsive HCU is significantly improved by Hcy lowering therapy [1,6] compliance with treatment is difficult and often poor. In order to improve treatment for HCU, there is a pressing need for a greater understanding of the pathogenic mechanisms that underlie this disease.

Cytokines and chemokines are soluble proteins or glycoproteins produced by multiple cell types that function as cellular communication molecules exerting pleiotropic regulatory effects. Most are secreted but some can be expressed on the cell membrane while others can be sequestered in the extracellular matrix and held or released according to biological demand [7]. The relative balance of pro and anti-inflammatory cytokine and chemokine expression is believed to play a significant role in the etiology of both thrombosis and atherogenesis [8]. In order to investigate the possible contribution of chronic inflammation to pathogenesis in HCU, we analyzed plasma cytokine and chemokine expression levels in a transgenic mouse model of the disease in the presence and absence of betaine treatment and compared these findings to those observed in control mice. Additionally, we performed an expanded analysis of cytokine and chemokine levels in human subjects with HCU in the presence and absence of conventional treatment. We observed that both mice and humans with HCU exhibit highly significant constitutive induction of multiple pro-inflammatory and chemotactic cytokines/chemokines while the expression levels of anti-inflammatory cytokines were unaffected. With the exception of tumor necrosis factor alpha (TNF- α), all of the effects of HCU upon cytokine expression in humans were effectively abolished by effective Hcy lowering therapy. Collectively, our findings indicate that HCU is a disease of

chronic inflammation where aberrant cytokine expression has the clear potential to contribute to multiple aspects of the disease and that targeted anti-inflammatory drugs have the potential to serve as a useful adjuvant therapeutic strategy.

2. Materials and methods

2.1. Animal studies

The generation of the human transgenic HO mouse model of HCU has been described previously [9]. For the experiments reported here, we used experimental groups consisting of 8 HO and 8 C57BL/6J control mice bred in house. Mice in both groups were male and aged between 3 and 4 months. All mice were maintained on standard chow (LabDietNIH5K67, PMI nutrition international, Brentwood, MO) and were weighed before plasma was taken. There was no significant difference in body weight between mice in any of the experimental groups. Trimethylglycine (betaine) (Sigma-Aldrich) was administered by dissolving this compound in drinking water (20 g/l) and was supplied ad libitum for one week. All experiments were approved by the University of Colorado Health Sciences Center institutional animal care and use committee and were performed according to the NIH standards for animal care and use.

2.2. Human subjects

Plasma samples were drawn from pyridoxine non-responsive HCU patients during their routine scheduled visits to the metabolic clinic at the Denver Children's Hospital. All subjects were consented for our IRB approved research protocol prior to the analysis of their plasma samples. HCU patients ranged between 5 and 32 years of age. Three of the untreated or poorly compliant patients had experienced at least one thrombotic event prior to our analysis. Of the five treated subjects, two were receiving cysteine supplementation (at either 90 mg per kg per day of cysteine or 17.7 mg per kg per day of cysteine) in addition to betaine therapy and protein restriction.

2.3. Genotype determinations

Mouse genotypes were determined initially by PCR analysis of genomic DNA obtained from tail snips as described previously [9]. The genotypes of all animals used in this study both before and after betaine treatment were confirmed by determination of tHcy levels in plasma samples obtained by non-lethal tail bleeding.

2.4. Thiols and methionine cycle metabolites

Plasma tHcy, methionine, total cysteine, dimethylglycine and methylglycine levels were determined from 20 μ l plasma samples as described previously [10,11].

2.5. Analysis of plasma C-reactive protein and cytokines

C-reactive protein (CRP) levels were determined in mouse plasma by ELISA (R and D systems, Minneapolis, MN.) using the manufacturer's standard protocol.

The concentrations of 14 cytokines and chemokines in mouse plasma samples were measured using a Bio-Plex protein array system (Bio-Rad, Hercules, CA), based on xMAP

technology (Luminex, Austin, TX). One 96-well multiplex panel (Bio-Plex Mouse Cytokine Panel, Bio-Rad catalog #: X600002071) was used to simultaneously quantify the plasma levels of IL-1 alpha (IL-1a), IL-1beta (IL-1b), IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12(p40), IL-1 receptor antagonist (IL-1RA), granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage inflammatory protein 2 (MIP-2, also known as keratinocyte chemoattractant), regulated upon activation, normal T-cell expressed, and secreted (RANTES) and TNF-a in 30 µl plasma samples using the manufacturer's standard protocol with minor modifications. Briefly, plasma samples were diluted 1:2, and recombinant cytokines were reconstituted and serially diluted to generate a standard curve (Mouse Serum Diluent Kit, Bio-Rad (catalog #171-304000)). Beads with capturing antibodies, standards and samples were added to a 96 wells microtiter plate (Millipore Corporation, Billerica, MA), and incubated for 2 h, before adding detection antibody and streptavidin-PE (Bio-Plex Reagent Kit, Bio-Rad). The plate was then analyzed with a Bio-Plex 200 System Workstation.

For human plasma samples a custom array was designed to assay a total of 16 cytokines including IL-1a, IL-1b, IL-1RA, IL-6, IL-8, IL-9, IL-10, IL-12(p70), IL-17, granulocyte colony stimulating factor (G-CSF), monocyte chemoattractant protein (MCP)-1, MIP-1alpha, MIP-1b, transforming growth factor-alpha (TGF-a), TNF-a; and fractalkine. The Luminex analyses were performed according to the manufacturer's standard protocol. To avoid the effect of any possible inter-assay variation, all samples compared statistically in both the human and mouse experiments were analyzed simultaneously on the same microtiter plate. Samples assayed in the luminex analysis were analyzed in duplicate to enhance precision. All duplicate samples were within 5% of each other. Values shown are the average of the duplicates.

To validate the results from the Luminex analyses. TNF-a in both mouse and human plasma samples were also analyzed by ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's standard protocol.

2.6. Statistical analysis

All values are presented as means $\pm$ SD. For comparisons, unpaired two-sided Student's *t*-tests were used. Differences between groups were considered significant at a *p* value of <0.05 . In the graphed data *, **, and *** denote *p* values of <0.05 , 0.01 and 0.001 respectively.

3. Results

3.1. HCU induces a chronic inflammatory state in a transgenic mouse model of the disease

The level of high sensitivity CRP in plasma is a well established and reliable marker of inflammation that has been shown to have considerable prognostic value for atherothrombosis of the coronary and cerebral vessels [12,13]. To test our hypothesis that HCU induces a chronic inflammatory state, we used an ELISA method to determine the basal plasma levels of CRP in wild type (WT) control mice and the previously described HO transgenic mouse model of HCU that was developed in our laboratory [9] (*n*=8 for both groups). To examine the possible ameliorating effect of tHcy lowering treatment, we also

examined the plasma CRP levels in a group of HO HCU mice (n=8) that had been treated with betaine as described in the materials and methods section. The betaine treatment decreased plasma tHcy levels from an average of 240.62 μ M (SD 62.3) to 46.5 μ M (SD 17.2, $p<0.0001$). In the CRP analysis, we observed a highly significant 7- fold induction of CRP in the HO HCU mice ($p<0.0001$) indicating that HCU induces a state of chronic inflammation (Fig. 1). The possible causative relationship between severely elevated Hcy and CRP was further supported by the observation that betaine treatment significantly decreased the observed plasma CRP level in HO HCU mice by approximately 50% although it remained significantly elevated compared to the normal control mice ($p<0.0001$) (Fig. 1).

3.2. The HO mouse model of HCU exhibits constitutive expression of multiple pro-inflammatory cytokines

The inflammatory response consists of the sequential release of cytokine and chemokine mediators and the recruitment of circulating leukocytes, which become activated at the inflammatory site and release further cytokine and chemokine mediators [14]. Under normal physiological conditions, the inflammatory response is resolved by the release of endogenous anti-inflammatory cytokines as well as the accumulation of intracellular negative regulatory factors. In order to further investigate the possible role of chronic inflammation in the pathogenesis of HCU, we simultaneously assayed the plasma levels of the pro-inflammatory cytokines IL-1a, IL-1b, IL-6, IL-12(p40), MIP-2, GM-CSF, TNF-a and the anti-inflammatory cytokines IL-1RA, IL-4 and IL-10 in plasma samples derived from wild type control mice (n=8, mean plasma tHcy=4.06 μ M, SD=0.69) and HO HCU mice (n=8, mean plasma tHcy=227.4 μ M, SD=52) using a luminex microbead platform as described in the Materials and methods section. As controls, our analysis also simultaneously assayed the expression levels of a number of cytokines that are not normally associated with inflammation namely, RANTES, IL-2, IL-3 and IL-5.

Our multiplex analysis of cytokine expression revealed highly significant 3 and 4-fold induction of the pro-inflammatory cytokines IL-1a, and IL-1b respectively (Fig. 2a and b) in the HO HCU mice compared to normal controls. ($p=0.0007$ and 0.0004 respectively). Similarly, the HO mice exhibited a 35-fold induction in the plasma level of the pro-inflammatory cytokine TNF-a compared to WT mice ($p<0.0001$) (Fig. 3a). Consistent with these findings, a TNF-a specific ELISA performed using plasma samples from all animals also showed an approximately 30-fold induction of this pro-inflammatory cytokine in the HO mice compared to wild type animals (data not shown). Notably, induction of IL-1a, IL-1b and TNF-a was observed in all of the HO mice that were investigated. In three of the 8 HO animals investigated, we observed relatively large scale induction of the proinflammatory cytokine IL-6. However, the complete absence of this cytokine from the remaining 5 animals of the HO group meant that the inherent variability of IL-6 expression in the HO mice precluded the achievement of statistical significance. We observed no significant difference in the plasma levels of the pro-inflammatory cytokines MIP-2, GM-CSF or IL-12 between the experimental groups (data not shown). Similarly, the expression levels of RANTES, IL-2, IL-3, IL-5 in the HO HCU mice did not differ significantly from those observed in the wild type control mice (data not shown) suggesting that HCU does not induce a generalized effect upon cytokine expression unrelated to inflammation.

Our multiplex expression analysis also determined that the expression levels of the anti-inflammatory cytokines IL-1RA, IL-4 and IL-10 were not significantly changed in the HO HCU mice relative to control mice. Our results indicate that HCU induces a chronic proinflammatory state that is prolonged at least in part by a failure to elicit a normalizing anti-inflammatory response.

3.3. Betaine treatment of HO HCU mice results in a significant decrease in pro-inflammatory cytokine expression

To investigate the effect of tHcy lowering therapy upon cytokine expression in HCU, we treated all of the mice in the HO group with betaine for one week, and then took plasma samples for both tHcy determination and cytokine profiling as described above.

Betaine treatment of these mice resulted in a significant reduction in plasma tHcy in the HO mice compared to the untreated HO HCU group. (mean=48.425 μ M, SD=21.1). HO HCU mice treated with betaine did not exhibit any significant difference in the plasma levels of MIP-2, GM-CSF, RANTES, IL-2, IL-3, IL-4, IL-5, IL-6, IL-12, or IL-1RA compared to either the WT mice or the untreated HO HCU mice. Strikingly, the lowering of tHcy in the HO mice was accompanied by effective normalization of the plasma levels of the pro-inflammatory cytokine IL-1b but not IL-1a (Fig. 1a and b). The plasma levels of TNF-a were significantly reduced ($p<0.0001$) but still remained significantly higher than those observed in the WT animals ($p<0.0001$) (Fig. 3a). We observed a similar 4.8-fold reduction in TNF-a when these samples were examined by ELISA (data not shown). Our results indicate that reducing tHcy with betaine treatment acts to significantly ameliorate the constitutive expression of pro-inflammatory cytokines induced by HCU and is consistent with the possibility that chronic inflammation contributes to the hypercoagulative phenotype previously observed in HO HCU mice.

3.4. Betaine treatment induces expression of the anti-inflammatory cytokine IL-10 in HO mice

Previous research has suggested that betaine might have anti-inflammatory properties independent of its role in lowering tHcy by serving as a methyl donor in the remethylation of this compound to methionine [15,16]. It is therefore conceivable, that betaine could exert protective effects in HCU by inducing the expression of anti-inflammatory cytokines. We observed that betaine treatment of the HO HCU animals induced a highly significant 17-fold induction in the plasma levels of IL-10 (Fig. 3b) compared to the untreated HO HCU mice ($p<0.0001$). This finding is particularly interesting given that numerous groups have reported on the highly potent protective effects of IL-10 in the context of IL-1 and TNF-a mediated atherogenesis, endothelial dysfunction and thrombogenesis [17].

3.5. Plasma cytokine profiling of human subjects with HCU reveals constitutive expression of pro-inflammatory cytokines and chemokines

Having observed a consistent pattern of constitutive expression of pro-inflammatory cytokines in our mouse model of HCU, we extended our analysis to investigate plasma cytokine expression patterns in human subjects with HCU in the presence and absence of conventional treatment. Our control group consisted of age and gender-matched normal

subjects with plasma tHcy between 2.3 and 3.6 μM (mean=2.78 μM , SD=0.622, n=5). Our second experimental group consisted of plasma samples from patients who were either untreated or poorly compliant. These subjects all exhibited plasma tHcy levels greater than 150 μM , (range 159 to 310.1 μM , mean=232.2 μM , SD=60.9, n=5). The third experimental group consisted of HCU patients who were receiving betaine treatment and were adhering to a methionine-restricted diet. These patients all exhibited a plasma tHcy level of less than or equal to 86.1 μM (range 24.5 to 86.1 μM , mean=50.6 μM , SD=25.7, n=5).

Guided by our results in HCU mice, we expanded our human luminex analysis panel to include IL-17 which functions as a proinflammatory cytokine acting synergistically with TNF- α and IL-1 [18] and in certain tissues, mediates the induction of TNF- α , IL-1, IL-6, G-CSF and GM-CSF expression [19]. Additionally, we included a number of chemokines that have previously been shown to be down stream targets of IL-1 α and β and/or TNF- α including IL-8 (which does not exist in mice), MCP-1, MIP-1 α , MIP-1 β and fractalkine that are known to mediate multiple cellular processes relevant to thrombosis, endothelial dysfunction and atherogenesis [17]. The plasma levels of the anti inflammatory cytokines IL-1RA and IL-10 were also determined.

In addition to these factors, we also investigated the plasma level of a number of cytokines that are ostensibly unconnected to inflammation including, granulocyte colony stimulating factor (G-CSF), IL-9 and TGF- α . G-CSF is a glycoprotein cytokine that is produced by macrophage and vascular endothelium and acts to induce the production of stem cells and granulocytes in the bone marrow and to regulate the survival and function of both neutrophils and their precursors [20]. IL-9 is produced by T-cells and specifically by CD4 $^{+}$ helper cells that acts as a regulator of a variety of hematopoietic cells but is known primarily for its effects upon mast cells [21]. TGF- α is a cytokine that due to its similarity to epidermal growth factor (EGF) and its binding to the EGF receptor is thought to function primarily as a growth factor playing a key role in epithelial development [22].

Our analysis revealed that for the normal subject controls, all of the cytokines assayed, with the exception of fractalkine (normal range 63.5–439 pg/mL but in our analysis, was found to be below the limits of detection), were within the normal range (Tables 1 and 2). We did not observe any significant change in IL-1 β (undetectable in all groups), IL-8, IL-1RA, MCP-1 or IL-10 in any of the experimental groups (Tables 1 and 2). Similar to our findings in HCU mice, our analysis revealed that HCU in human subjects induces highly significant constitutive expression of the pro-inflammatory cytokines IL-1 α (3.7-fold), IL-6 (72-fold), TNF- α (22-fold), IL-17 (induced from undetectable up to an average of 24.5 pg/mL, SD=12.4) and IL-12 (p70) (23-fold) compared to the normal controls. Similarly, we also observed significant constitutive expression of the chemotactic chemokines, fractalkine (undetectable in the normal controls), MIP- 1 α (2-fold), and MIP-1 β (2-fold) in the HCU patients. Interestingly, we also observed a significant induction of IL-9, TGF- α (both of which were undetectable in all of the control samples) and G-CSF (2-fold) in the HCU patients. With the exception of TNF- α , all of the cytokines that were induced in the untreated HCU group were found to be within the normal range in the HCU plus treatment group indicating that Hcy lowering therapy acts to significantly ameliorate the HCU induced pro-inflammatory state.

3.6. Plasma levels of TNF- α remain significantly elevated in human HCU patients even in the presence of Hcy lowering therapy

The observed failure of betaine treatment to normalize plasma TNF- α in patients with HCU in our luminex experiment prompted us to corroborate this finding by independently determining the TNF- α levels in the plasma of all of the human patients using an ELISA method (Fig. 4). In this experiment, we observed that TNF- α was induced some 30-fold compared to the control group ($p < 0.0001$). The treated HCU group showed a statistically significant 3.4-fold reduction compared to the untreated HCU group ($p = 0.001$) but the level remained significantly elevated compared to the normal control group ($p = 0.008$). Collectively, our results in both HCU mice and patients indicate that conventional Hcy lowering therapy does not completely normalize the plasma levels of this pro-inflammatory cytokine.

3.7. Hcy lowering therapy affects plasma levels of multiple metabolites in HCU

Although significantly lowering tHcy results in effective normalization of the expression levels of most pro-inflammatory cytokines in both mice and humans with HCU, it should be noted that in addition to Hcy, betaine treatment acts to modify the plasma and tissue levels of a number of other metabolites as well. Previous work with the HO mouse model of HCU showed that untreated HCU mice had significantly increased plasma methionine, AdoMet and AdoHcy and decreased cysteine levels compared to wild type controls [9]. Treatment of these mice with betaine resulted in a significant decrease in plasma AdoMet and AdoHcy and a significant increase in plasma methionine, cysteine, dimethylglycine and methylglycine compared to untreated HCU animals [9]. Similarly, in this study, we observed significant increases in plasma methionine, cysteine, methylglycine and dimethylglycine in HCU patients receiving conventional therapy compared to untreated HCU patients (Fig. 5). In this study we did not determine plasma AdoMet and AdoHcy levels in human HCU.

4. Discussion

In this study we have used a combination of ELISAs and a multiplex micro-bead array system to investigate the cytokine profiles induced by HCU in both human patients and an animal model of the disease in the presence and absence of Hcy lowering therapy. Both the animal model and the patients exhibited highly significant induction of multiple proinflammatory cytokines/chemokines while the expression levels of anti-inflammatory cytokines were essentially unaffected. Results in the HO HCU mouse model while not identical, were broadly consistent with the results in humans indicating that the HO model effectively recapitulates this aspect of the human disease and will be a useful tool to examine both the mechanism/s that induce this constitutive inflammation and possible novel adjuvant therapies for this disease.

Previous work using tissue culture models has suggested possible cytokine involvement in the pathogenesis of HCU [23–25], but to our knowledge, a detailed investigation of this aspect of the HCU phenotype has not been performed previously in either patients or an animal model of the disease. While much broader in scope than previous studies, our results

concerning the elevations in IL-1a and b, TNF-a and IL-6 are in broad agreement with those reported in the various tissue culture models [23–25].

Pro-inflammatory cytokines are generally not constitutively produced but are typically produced in response to injury where they induce the activation of a wide range of target cell types in order to mobilize the immune system to distinguish between self and non-self and to promote reparative processes by mediating the inflammatory response. While this is essentially a protective mechanism, chronic inappropriate expression of the inflammatory response can be deleterious and in the past few years, inflammation has emerged as a major pathogenic determinant in both thrombosis and atherosclerotic lesion development, both of which are frequent clinical sequelae in HCU. An abundance of data exists supporting the idea that elevated pro-inflammatory cytokines contribute directly to cardiovascular disease and that the endothelium is a major target of cytokine signals derived from different vascular cells. It has previously been demonstrated that endothelial activation, an early step in vascular dysfunction, can be induced by TNF-a, IL-1a and b and IL-6 [26]. Previous work in mouse models of CBS impairment proposed a pathogenic role for paradoxical vasoconstriction as a consequence of decreased bioavailability of nitric oxide [27]. In this context, it is interesting to note that TNF-a has been demonstrated to directly decrease levels of constitutive nitric oxide synthase mRNA in human endothelial cells [28] and has also been shown to selectively diminish the ability of arterial rings to relax in response to the endothelium-dependent vasodilator acetylcholine [29]. In addition to TNF-a, both IL-6 and G-CSF have been shown to exhibit opposing regulatory roles in the modulation of vascular tone. In these studies, cytokine-mediated relaxation (G-CSF) or constriction (TNF-a and IL-6) were inhibited by blockers of endothelial-derived nitric oxide and endothelin respectively [30]. Consistent with these findings, it has been reported that patients with congestive heart failure exhibit a correlation between plasma levels of IL-6 and TNF-a and impaired endothelium-dependent vasodilation of the brachial artery and vein [31,32]. Collectively, our results indicate that the cytokine profiles observed in HCU have the potential to contribute to endothelial vasodilator dysfunction.

Previous research indicates that the pro-inflammatory cytokines observed in our analysis to be induced by HCU, have the potential to contribute to the thrombotic phenotype of HCU by promoting coagulation and also by inhibiting endogenous anti-coagulant function. Tissue factor (TF), is a major initiator of extrinsic coagulation that rapidly induces coagulation upon contact with blood. TF is normally located in the extra-vascular space, typically on the surface of fibroblast cells. Pro-inflammatory cytokines such as TNF-a and IL-1a and b have previously been shown to induce the expression of TF on endothelial cells and monocytes [33]. Thus TNF-a and IL-1a and b mediated induction of TF expression in an intra-vascular milieu constitutes a possible mechanism by which constitutive inflammation could predispose towards thrombogenesis in HCU. Inflammation can also affect systemic hemostatic activity by IL-6-mediated stimulation of hepatocytes to produce acute-phase reactants [34].

In addition to these possible mechanisms, pro-inflammatory cytokines have the potential to promote coagulation by impairing the function of the protein C anticoagulant pathway which is initiated when thrombin binds to thrombomodulin (TM). TM is expressed

constitutively on endothelium and it has been previously demonstrated that both TNF- α and IL-1 α and β cause a slow loss of TM and endothelial cell protein C receptor from the surface of endothelial cells [35]. In addition to these possible mechanisms, previous work has shown that IL-6 can stimulate the formation of new platelets with increased sensitivity to thrombin activation and increased procoagulant activity [36]. In this context, it is interesting to note that MIP-1 α , which we have observed to be induced in HCU, is an α granule chemokine that is both a marker for platelet activation and a mediator of further activation of other platelets [37]. We have previously demonstrated a hypercoagulative phenotype in the HO mouse model using a tail bleeding assay [9]. This assay is strongly influenced by platelet aggregation and this aspect of hemostasis in HCU will be the subject of future investigations in our laboratory.

With regard to the role of cytokine involvement in atherogenesis in HCU, it is now well-established that pro-inflammatory cytokines participate in the disease process from early lesion to vulnerable plaque formation. The most prominent cells that invade in evolving lesions are monocyte-derived macrophages and T-lymphocytes both of which produce a wide array of soluble pro-inflammatory cytokines/chemokines which are critically important in the initiation and perpetuation of the disease. Previous work has demonstrated that both IL-1 isoforms promote the adhesion of neutrophils, monocytes, T-cells, and B-cells in the vasculature by enhancing the expression of adhesion molecules such as the intercellular adhesion molecule CAM-1 and the endothelial-leukocyte adhesion molecule ELAM and are consistently present in all vessels with sclerotic histopathologic changes [38]. TNF- α was recently implicated in atherogenesis by comparative analysis of early fatty-streak lesion in apoE and TNF- α double mutant mice. This study reported that deletion of TNF- α acts to retard fatty-streak lesion formation by down regulating the expression of multiple pro-atherosclerotic inflammatory factors [39]. Fractalkine (CX3CL1), which was found to be strongly induced in the human HCU analysis, is a downstream regulatory target of IL-1 α and β and TNF- α and has been strongly linked to atherogenesis. This chemokine plays a regulatory role in cell trafficking and endothelial cell adhesion by serving as a chemoattractant at inflammatory sites and is almost invariably expressed in atherosclerotic plaques [40,41]. Other IL-1/TNF- α targets that were found to be induced by HCU in humans with the potential to contribute to the cardiovascular phenotype include MIP-1 α (CCL3) and MIP-1 β (CCL4). These chemokines are highly related members of the CC chemokine subfamily that orchestrate acute and chronic inflammatory host responses at sites of injury or infection mainly by recruiting proinflammatory cells and regulating cell adhesion to the endothelium[42].

The potential pathogenic contribution of the observed cytokine expression profile in both mice and humans with HCU is not limited to the cardiovascular sequelae. Both IL-1 and TNF- α has been associated with promoting osteoporosis by activating osteoclast mediated bone resorption [43]. This latter possibility would provide an explanation for the fact that osteoporosis is absent from children with Marfan syndrome but is a common feature in children with HCU. It is interesting to note that Hcy lowering therapy in both HCU mice and human patients did not completely normalize plasma TNF- α levels. Although no definitive study has ever been carried out, anecdotal evidence suggests that HCU-induced osteoporosis is not completely corrected by existing treatments, an observation that is consistent with our

data regarding TNF- α in individuals with HCU. Our findings suggest that patients that continue to experience reduced bone density while receiving conventional therapy might benefit from adjuvant anti-inflammatory therapy.

In addition to contributing to the cardiovascular and connective tissue sequelae, it is also possible that the cytokine profiles in HCU described here could contribute to the cognitive impairment that is frequently observed in this disease. IL-1 has been observed in the CNS both as the product of local synthesis and also due to the direct transport of IL-1 through the blood–brain barrier by means of a saturable carrier system [44]. IL-1 has been shown to modulate the electrophysiological behavior of neurons and to activate the serotonergic system [45]; similarly, Brett et al. have used a transgenic mouse model to express IL-6 in brain astrocytes and demonstrated a subsequent extensive break down of the blood brain barrier [46]. Intra-cerebral over-expression of IL-6 in transgenic mice has been shown to be associated with neurological syndromes the severity of which, correlate with the levels of IL-6 expression suggesting a direct pathogenic role of IL-6 in inflammatory neurodegenerative CNS diseases [47,48].

In terms of HCU induced cognitive impairment, not all of the cytokines that we found to be induced in human HCU plasma are deleterious and a number have the potential to serve protective roles. TGF- α stimulates neural cell proliferation in the adult injured brain [49] and IL-9/IL-9 receptor signaling has been shown to selectively protect cortical neurons against apoptosis [50]. Similarly, Boehme et al. have shown that fractalkine may function as a survival factor for primary microglial cells while G-CSF acts to promote neurogenesis and counteracts multiple pro-apoptotic signals [51].

Our findings prompt the question as to why HCU should induce chronic inflammation? The most likely explanation is that the severe elevations in the plasma and tissue concentrations of the pro-oxidant Hcy and the concomitant diminution in the endogenous synthesis of the anti-oxidant compounds cysteine and glutathione, caused by inactivation of CBS, result in significant oxidative stress. A large body of evidence exists indicating that changes in thiol homeostasis and redox signaling decisively contribute to amplification of the inflammatory cascade through mitogen activated protein kinase (MAP kinase) pathways [52]. This possibility is consistent with our observation that reducing Hcy and increasing cysteine via betaine treatment resulted in significant decreases in nearly all of the pro-inflammatory cytokines measured in both the HO mouse model and the human HCU patients. However, it is conceivable that betaine exerts anti-inflammatory effects independent of its role in lowering Hcy. Previous work investigating the effects of betaine supplementation in aging rats with normal Hcy levels found that this treatment ameliorated the activation of the nuclear factor-kappaB transcription factor which has previously been shown to regulate the induction of a number of the pro-inflammatory cytokines that we have observed to be constitutively expressed in HCU [53].

Other metabolite changes present in Hcy mice and humans are less likely to cause this inflammatory profile. As methionine and methylglycine levels are increased in untreated HCU patients with elevated pro-inflammatory cytokines and are further increased by betaine treatment, we can probably rule out a role for these compounds in the regulation of cytokine

expression in HCU. Dimethylglycine levels do not differ significantly between the control subjects and untreated HCU patients; hence this compound is unlikely to be playing a role in the induction of inflammatory cytokines. However, since plasma levels of this compound were elevated with betaine treatment, we cannot completely exclude the possibility of a previously unsuspected anti-inflammatory role for this compound. Similarly, as cysteine was decreased in the untreated HCU subjects compared to normal controls and was significantly increased in the treated HCU subjects, we cannot rule out a role for cysteine or AdoMet and AdoHcy in regulating the expression levels of the cytokines investigated. Experiments designed to investigate the effects of antioxidant therapy upon the cytokine profile in the HO mouse model are currently being performed in our laboratory. A formal clinical study of these cytokines in humans and their response to a targeted treatment is currently recruiting.

Acknowledgments

K.N.M gratefully acknowledges financial support from the Jerome Lejeune Foundation, Special Products U.K., the William R. Hummel Homocystinuria Research Fund and research award 6703 from the Denver Children's Hospital. A.K.K. is a St. Baldrick's Foundation Scholar. C.F. and J.V.H are supported by grants from the University of Colorado Clinical and Translational Science Institute and from the Walter S. and Licienne Driskill Foundation. The authors gratefully acknowledge the assistance of the University of Colorado Clinical and Translational Science Institute in performing the human luminex cytokine analysis.

Abbreviations

HCU	classical homocystinuria
CRP	C-reactive protein
CBS	cystathionine beta-synthase
CGL	cystathionine gamma-lyase
CVD	cardiovascular disease
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
Hcy	homocysteine
IL	interleukin
IL-1a	IL-1 alpha
IL-1b	IL-1beta
IL-1RA	IL-1 receptor antagonist
MCP	monocyte chemoattractant protein
MIP	macrophage inflammatory protein
RANTES	regulated upon activation, normal T-cell expressed, and secreted
AdoMet	S-adenosylmethionine
AdoHcy	S-adenosylhomocysteine
TF	tissue factor

TM	thrombomodulin
tHcy	total homocysteine
TGF-α	transforming growth factor- α
TNF-α	tumor necrosis factor α

References

1. Mudd, SH.; Levy, HL.; Kraus, JP. Disorders of transsulfuration. In: Scriver, CR.; Beaudet, AL.; Sly, WS.; Valle, D.; Childs, B.; Kinzler, K.; Vogelstein, B., editors. *The Metabolic and Molecular Bases of Inherited Disease*. McGraw-Hill; New York: 2001. p. 2007-2056.
2. Selhub J. Mild hyperhomocysteinemia and arterial occlusive disease. *Haematologica*. 1997; 82:129–132. [PubMed: 9175312]
3. D'Angelo A, Selhub J. Homocysteine and thrombotic disease. *Blood*. 1997; 90:1–11. [PubMed: 9207431]
4. Lonn E, Yusuf S, Arnold MJ, Sheridan P, Pogue J, Micks M, McQueen MJ, Probstfield J, Fodor G, Held C, Genest J Jr. Homocysteine lowering with folic acid and B vitamins in vascular disease. *N Engl J Med*. 2006; 354:1567–1577. [PubMed: 16531613]
5. Bona KH, Njolstad I, Ueland PM, Schirmer H, Tverdal A, Steigen T, Wang H, Nordrehaug JE, Arnesen E, Rasmussen K. Homocysteine lowering and cardiovascular events after acute myocardial infarction. *N Engl J Med*. 2006; 354:1578–1588. [PubMed: 16531614]
6. Mudd SH, Skovby F, Levy HL, Pettigrew KD, Wilcken B, Pyeritz RE, Andria G, Boers GHJ, Bromberg IL, Cerone R, Fowler B, Grobe H, Schmidt H, Schweitzer L. The natural history of homocystinuria due to cystathionine b-synthase deficiency. *Am J Hum Genet*. 1985; 37:1–31. [PubMed: 3872065]
7. Hanada T, Yoshimura A. Regulation of cytokine signaling and inflammation. *Cytokine Growth Factor Rev*. 2002; 13:413–421. [PubMed: 12220554]
8. Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med*. 1999; 340:115–126. [PubMed: 9887164]
9. Maclean KN, Sikora J, Kozich V, Jiang H, Greiner LS, Kraus E, Krijt J, Overdier KH, Collard R, Brodsky GL, Meltesen L, Crnic LS, Allen RH, Stabler SP, Elleder M, Rozen R, Patterson D, Kraus JP. A novel transgenic mouse model of CBS-deficient homocystinuria does not incur hepatic steatosis or fibrosis and exhibits a hypercoagulable phenotype that is ameliorated by betaine treatment. *Mol Genet Metab*. 2010; 101:153–162. [PubMed: 20638879]
10. Allen RH, Stabler SP, Lindenbaum J. Serum betaine, *N,N*-dimethylglycine and *N*-methylglycine levels in patients with cobalamin and folate deficiency and related inborn errors of metabolism. *Metabolism*. 1993; 42:1448–1460. [PubMed: 7694037]
11. Stabler SP, Lindenbaum J, Savage DG, Allen RH. Elevation of serum cystathionine levels in patients with cobalamin and folate deficiency. *Blood*. 1993; 81:3404–3413. [PubMed: 8507876]
12. Ridker PM, Hennekens CH, Buring JE, Rifai N. C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. *N Engl J Med*. 2000; 342:836–843. [PubMed: 10733371]
13. Haverkate F, Thompson SG, Pyke SD, Gallimore JR, Pepys MB. Production of C-reactive protein and risk of coronary events in stable and unstable angina. European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group. *Lancet*. 1997; 349:462–466. [PubMed: 9040576]
14. Kishimoto T, Taga T, Akira S. Cytokine signal transduction. *Cell*. 1994; 76:253–262. [PubMed: 8293462]
15. Abdelmalek MF, Angulo P, Jorgensen RA, Sylvestre PB, Lindor KD. Betaine, a promising new agent for patients with nonalcoholic steatohepatitis: results of a pilot study. *Am J Gastroenterol*. 2001; 96:2711–2717. [PubMed: 11569700]

16. Maclean KN, Sikora J, Kozich V, Jiang H, Greiner LS, Kraus E, Krijt J, Crnic LS, Allen RH, Stabler SP, Elleder M, Kraus JP. Cystathionine beta-synthase null homocystinuric mice fail to exhibit altered hemostasis or lowering of plasma homocysteine in response to betaine treatment. *Mol Genet Metab.* 2010; 101:163–171. [PubMed: 20638882]
17. Kofler S, Nickel T, Weis M. Role of cytokines in cardiovascular diseases: a focus on endothelial responses to inflammation. *Clin Sci (Lond).* 2005; 108:205–213. [PubMed: 15540988]
18. Miossec P, Korn T, Kuchroo VK. Interleukin-17 and type 17 helper T cells. *N Engl J Med.* 2009; 361:888–898. [PubMed: 19710487]
19. Aggarwal S, Gurney AL. IL-17: prototype member of an emerging cytokine family. *J Leukoc Biol.* 2002; 71:1–8. [PubMed: 11781375]
20. Demetri GD, Griffin JD. Granulocyte colony-stimulating factor and its receptor. *Blood.* 1991; 78:2791–2808. [PubMed: 1720034]
21. Renauld JC, Houssiau F, Louahed J, Vink A, Van Snick J, Uyttenhove C. Interleukin-9. *Adv Immunol.* 1993; 54:79–97. [PubMed: 8379467]
22. Derynck R. The physiology of transforming growth factor-alpha. *Adv Cancer Res.* 1992; 58:27–52. [PubMed: 1546559]
23. Poddar R, Sivasubramanian N, DiBello PM, Robinson K, Jacobsen DW. Homocysteine induces expression and secretion of monocyte chemoattractant protein-1 and interleukin-8 in human aortic endothelial cells: implications for vascular disease. *Circulation.* 2001; 103:2717–2723. [PubMed: 11390343]
24. Dalal S, Parkin SM, Homer-Vanniasinkam S, Nicolaou A. Effect of homocysteine on cytokine production by human endothelial cells and monocytes. *Ann Clin Biochem.* 2003; 40:534–541. [PubMed: 14503991]
25. Su SJ, Huang LW, Pai LS, Liu HW, Chang KL. Homocysteine at pathophysiologic concentrations activates human monocyte and induces cytokine expression and inhibits macrophage migration inhibitory factor expression. *Nutrition.* 2005; 21:994–1002. [PubMed: 16157236]
26. Pober JS. Immunobiology of human vascular endothelium. *Immunol Res.* 1999; 19:225–232. [PubMed: 10493176]
27. Eberhardt RT, Forgiione MA, Cap A, Leopold JA, Rudd MA, Trolliet M, Heydrick S, Stark R, Klings ES, Moldovan NI, Yaghoubi M, Goldschmidt-Clermont PJ, Farber HW, Cohen R, Loscalzo J. Endothelial dysfunction in a murine model of mild hyperhomocyst(e)inemia. *J Clin Invest.* 2000; 106:483–491. [PubMed: 10953023]
28. Yoshizumi M, Perrella MA, Burnett J Jr, Lee ME. Tumor necrosis factor downregulates an endothelial nitric oxide synthase mRNA by shortening its half-life. *Circ Res.* 1993; 73:205–209. [PubMed: 7685252]
29. Aoki N, Siegfried M, Lefer AM. Anti-EDRF effect of tumor necrosis factor in isolated, perfused cat carotid arteries. *Am J Physiol.* 1989; 256:H1509–H1512. [PubMed: 2785770]
30. Iversen PO, Nicolaysen A, Kvernebo K, Benestad HB, Nicolaysen G. Human cytokines modulate arterial vascular tone via endothelial receptors. *Pflugers Arch.* 1999; 439:93–100. [PubMed: 10651005]
31. Katz SD, Rao R, Berman JW, Schwarz M, Demopoulos L, Bijou R, LeJemtel TH. Pathophysiological correlates of increased serum tumor necrosis factor in patients with congestive heart failure. Relation to nitric oxide-dependent vasodilation in the forearm circulation. *Circulation.* 1994; 90:12–16. [PubMed: 8025986]
32. Vanderheyden M, Kersschot E, Paulus WJ. Pro-inflammatory cytokines and endothelium-dependent vasodilation in the forearm. Serial assessment in patients with congestive heart failure. *Eur Heart J.* 1998; 19:747–752. [PubMed: 9717008]
33. Shebuski RJ, Kilgore KS. Role of inflammatory mediators in thrombogenesis. *J Pharmacol Exp Ther.* 2002; 300:729–735. [PubMed: 11861775]
34. Tousoulis D, Davies G, Stefanadis C, Toutouzas P, Ambrose JA. Inflammatory and thrombotic mechanisms in coronary atherosclerosis. *Heart.* 2003; 89:993–997. [PubMed: 12923007]
35. Sohn RH, Deming CB, Johns DC, Champion HC, Bian C, Gardner K, Rade JJ. Regulation of endothelial thrombomodulin expression by inflammatory cytokines is mediated by activation of nuclear factor-kappa B. *Blood.* 2005; 105:3910–3917. [PubMed: 15677570]

36. Esmon CT. Possible involvement of cytokines in diffuse intravascular coagulation and thrombosis. *Baillieres Best Pract Res Clin Haematol*. 1999; 12:343–359. [PubMed: 10856974]
37. Gear AR, Camerini D. Platelet chemokines and chemokine receptors: linking hemostasis, inflammation, and host defense. *Microcirculation*. 2003; 10:335–350. [PubMed: 12851650]
38. Barksby HE, Lea SR, Preshaw PM, Taylor JJ. The expanding family of interleukin-1 cytokines and their role in destructive inflammatory disorders. *Clin Exp Immunol*. 2007; 149:217–225. [PubMed: 17590166]
39. Xiao N, Yin M, Zhang L, Qu X, Du H, Sun X, Mao L, Ren G, Zhang C, Geng Y, An L, Pan J. Tumor necrosis factor- α deficiency retards early fatty-streak lesion by influencing the expression of inflammatory factors in apoE-null mice. *Mol Genet Metab*. 2009; 96:239–244. [PubMed: 19157944]
40. Truman LA, Ford CA, Pasikowska M, Pound JD, Wilkinson SJ, Dumitriu IE, Melville L, Melrose LA, Ogden CA, Nibbs R, Graham G, Combadiere C, Gregory CD. CX3CL1/fractalkine is released from apoptotic lymphocytes to stimulate macrophage chemotaxis. *Blood*. 2008; 112:5026–5036. [PubMed: 18799722]
41. Garcia GE, Xia Y, Chen S, Wang Y, Ye RD, Harrison JK, Bacon KB, Zerwes HG, Feng L. NF- κ B-dependent fractalkine induction in rat aortic endothelial cells stimulated by IL-1 β , TNF- α , and LPS. *J Leukoc Biol*. 2000; 67:577–584. [PubMed: 10770292]
42. Lukacs NW, Strieter RM, Elner VM, Evanoff HL, Burdick M, Kunkel SL. Intercellular adhesion molecule-1 mediates the expression of monocyte-derived MIP-1 α during monocyte–endothelial cell interactions. *Blood*. 1994; 83:1174–1178. [PubMed: 7906962]
43. Mundy GR. Osteoporosis and inflammation. *Nutr Rev*. 2007; 65:S147–S151. [PubMed: 18240539]
44. Banks WA, Ortiz L, Plotkin SR, Kastin AJ. Human interleukin (IL) 1 α , murine IL-1 α and murine IL-1 β are transported from blood to brain in the mouse by a shared saturable mechanism. *J Pharmacol Exp Ther*. 1991; 259:988–996. [PubMed: 1762091]
45. Gemma C, Ghezzi P, De Simoni MG. Activation of the hypothalamic serotonergic system by central interleukin-1. *Eur J Pharmacol*. 1991; 209:139–140. [PubMed: 1726086]
46. Brett FM, Mizisin AP, Powell HC, Campbell IL. Evolution of neuropathologic abnormalities associated with blood–brain barrier breakdown in transgenic mice expressing interleukin-6 in astrocytes. *J Neuropathol Exp Neurol*. 1995; 54:766–775. [PubMed: 7595649]
47. Steffensen SC, Campbell IL, Henriksen SJ. Site-specific hippocampal pathophysiology due to cerebral overexpression of interleukin-6 in transgenic mice. *Brain Res*. 1994; 652:149–153. [PubMed: 7953712]
48. Campbell IL, Abraham CR, Masliah E, Kemper P, Inglis JD, Oldstone MB, Mucke L. Neurologic disease induced in transgenic mice by cerebral overexpression of interleukin 6. *Proc Natl Acad Sci U S A*. 1993; 90:10061–10065. [PubMed: 7694279]
49. Fallon J, Reid S, Kinyamu R, Opole I, Opole R, Baratta J, Korc M, Endo TL, Duong A, Nguyen G, Karkehabadhi M, Twardzik D, Patel S, Loughlin S. In vivo induction of massive proliferation, directed migration, and differentiation of neural cells in the adult mammalian brain. *Proc Natl Acad Sci U S A*. 2000; 97:14686–14691. [PubMed: 11121069]
50. Fontaine RH, Cases O, Lelievre V, Mesples B, Renaud JC, Loron G, Degos V, Dournaud P, Baud O, Gressens P. IL-9/IL-9 receptor signaling selectively protects cortical neurons against developmental apoptosis. *Cell Death Differ*. 2008; 15:1542–1552. [PubMed: 18551134]
51. Boehme SA, Lio FM, Maciejewski-Lenoir D, Bacon KB, Conlon PJ. The chemokine fractalkine inhibits Fas-mediated cell death of brain microglia. *J Immunol*. 2000; 165:397–403. [PubMed: 10861077]
52. Janssen YM, Van Houten B, Borm PJ, Mossman BT. Cell and tissue responses to oxidative damage. *Lab Invest*. 1993; 69:261–274. [PubMed: 8377469]
53. Go EK, Jung KJ, Kim JY, Yu BP, Chung HY. Betaine suppresses proinflammatory signaling during aging: the involvement of nuclear factor- κ B via nuclear factor-inducing kinase/I κ B kinase and mitogen-activated protein kinases. *J Gerontol A Biol Sci Med Sci*. 2005; 60:1252–1264. [PubMed: 16282556]
54. Maclean KN, Gaustadnes M, Oliveriusova J, Janosik M, Kraus E, Kozich V, Kery V, Skovby F, Rudiger N, Ingerslev J, Stabler SP, Allen RH, Kraus JP. High homocysteine and thrombosis

without connective tissue disorders are associated with a novel class of cystathionine beta-synthase (CBS) mutations. Hum Mutat. 2002; 19:641–655. [PubMed: 12007221]

Fig. 1.

HO HCU mice exhibit elevated levels of plasma CRP that responds to Hcy lowering therapy with betaine. Plasma CRP levels were determined in WT control mice and HO mice in the presence and absence of one week of betaine treatment by ELISA as described in the Materials and methods section. Values shown represent the mean and SD derived from 8 mice per group. Plasma tHcy was measured for each group and the values observed are shown as mean and SD in each respective bar of the graph. In this figure and all subsequent graphs presented here *, **, and *** denote p values of <0.05, 0.01 and 0.001 respectively.

Fig. 2. HCU induces the expression of the pro-inflammatory cytokines IL-1a and IL-1b in HO mice compared to WT controls. Plasma levels of (A) IL-1a and (B) IL-1b in HO HCU mice plus and minus betaine and untreated WT mice. Plasma cytokine levels were determined by luminex analysis as described in the Materials and methods section. Values shown represent the mean and SD derived from 8 mice per group.

Fig. 3. HCU induces expression of the pro-inflammatory cytokine TNF-a. Plasma levels of (A) TNF-a and (B) IL-10 in HO HCU mice. Plasma cytokine levels were determined by luminex analysis as described in the Materials and methods section. Values shown represent the mean and SD derived from 8 mice per group.

Fig. 4. TNF- α levels are significantly increased in untreated/poorly compliant human HCU patients and only partially normalized by conventional treatment. Plasma TNF- α levels were determined by ELISA as described in the Materials and methods. Values shown represent the mean and SD derived from 5 subjects per group.

Fig. 5. Betaine treatment and methionine restriction results in significant alterations in the plasma levels of methionine, cysteine, methylglycine and dimethylglycine in human subjects with HCU. Plasma levels of (A) methionine and total cysteine and (B) methylglycine and dimethylglycine in control subjects and HCU patients in the presence and absence of conventional treatment. Values shown represent the mean and SD derived from 5 subjects per group.

Table 1

Pro-inflammatory cytokine expression in human HCU.

	Hcy μM	IL-1a pg/mL	IL-1b pg/mL	IL-6 pg/mL	IL-8 pg/mL	IL-12p(70) pg/mL	IL-17 pg/mL	TNF-α pg/mL	MIP-1a pg/mL	MIP-1b pg/mL	MCP-1 pg/mL	Fractalkine pg/mL
Normal range	2–13.9	0–200	0–31.7	0–39	0.4–81	0–59	0–10	0.47–6.42	0–253	3.1–118.0	63–656	63.5–439
Control	2.78 (0.62)	187 (8.41)	0.0	0.66 (0.68)	5.7 (2.57)	0.8 (1.79)	0.0	2.4 (1.61)	64.5 (28.1)	64.3 (20.7)	641.32 (67.7)	0.0
HCU	232.2 (60.9)	694 (283)	0.0	47.84 (24)	11.58 (10.3)	18.5 (12.7)	24.5 (12.4)	54.6 (8.8)	120.4 (40.7)	122.9 (29.2)	508.4 (261.0)	746.3 (634.0)
HCU + treatment	50.6 (25.7)	119.8 (89.2)	0.0	0.84 (1.31)	7.8 (7.6)	0.0	1.5 (2.1)	15.4 (5.6)	26.8 (18.7)	32.0 (24.0)	641.7 (424.0)	36.0 (25.6)
Control vs. HCU p-value	<0.0001	0.0039	ND	0.0072	0.25 NS	0.015	0.0022	<0.0001	0.034	0.0064	0.30 NS	0.03
Control vs. HCU + treatment p-value	0.003	0.132 NS	ND	0.792 NS	0.57 NS	0.34 NS	0.143 NS	0.0011	0.037	0.052 NS	0.9 NS	0.0137
HCU vs. HCU + treatment p-value	0.0003	0.0025	ND	0.0024	0.527 NS	0.016	0.0034	<0.0001	0.0016	0.0007 NS	0.5659 NS	0.0368

Values shown represent the average plasma value derived from 5 subjects per group with the standard deviation given in parentheses. Normal range for tHcy determined from 80 normal subjects aged 18–65 years [54]. Normal range for cytokines defined by the assay manufacturer (Bio-Rad, Hercules, CA). ND, not determined; NS, not significant.

Table 2

Anti-inflammatory and non-inflammatory cytokine expression in human HCU.

	IL-1RA pg/mL	IL-9 pg/mL	IL-10 pg/mL	G-CSF pg/mL	TGF- α pg/mL
Normal range	0–167	0–75	0.25–102.00	0.99–22.20	0–25
Control	0.0	0.0	9.76 (0.963)	15.98 (1.8)	0.0
HCU	0.0	440.48 (25.9)	8.78 (0.829)	28.9 (6.39)	5.22 (4.39)
HCU + treatment	0.0	1.98 (4.43)	8.72 (2.64)	13.4 (2.14)	0.64 (0.9)
Control vs. HCU p-value	ND	<0.0001	0.12 NS	0.0024	0.0289
Control vs. HCU + treatment p-value	ND	0.346 NS	0.4314 NS	0.08 NS	0.3504 NS
HCU vs. HCU + treatment p-value	ND	<0.0001	0.962 NS	0.0009	0.0429

Values shown represent the average plasma value derived from 5 subjects per group with the standard deviation given in parentheses. Normal range for tHcy determined from 80 normal subjects aged 18–65 years [54]. Normal range for cytokines defined by the assay manufacturer (Bio-Rad, Hercules, CA). ND, not determined. NS, not significant.