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Effect of feeding a high fat diet on hydrogen sulfide (H₂S) metabolism in the mouse

Meng Teng Peh¹, Azzahra Binti Anwar¹, David S.W. Ng¹, Mohamed Shirhan Bin Mohamed Atan¹, Srinivasan Dinesh Kumar² and Philip K. Moore¹

¹Neurobiology Group, Life Sciences Institute and Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597; ²Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore 637553.

Short title: Hydrogen sulfide and fat diet

Author for correspondence:
Professor P.K. Moore, address as above.
Tel: 65-66011669
Fax: 65-6872 0830
E-mail: dprmpk@nus.edu.sg.
Abstract

Hydrogen sulfide (H$_2$S) has complex effects in inflammation. Both pro- and anti-inflammatory actions of this gas have been reported and recent work suggests that a deficiency of H$_2$S occurs in, and may contribute to, the inflammation which underpins atherosclerotic disease. Mice fed a high fat diet exhibit chronic, low grade systemic inflammation in the absence of frank atherosclerosis. In this study we determined whether H$_2$S metabolism was altered in mice fed a high fat diet for up to 16 weeks. Ex vivo biosynthesis of H$_2$S was reduced in liver, kidney and lung of mice fed a high fat diet. Western blotting revealed deficiency of cystathionine $\gamma$ lyase (CSE) in liver and lung with increased expression of cysthionine $\beta$ synthetase (CBS) in liver and kidney. Expression of the third H$_2$S synthesising enzyme, 3-mercaptopropruvate sulfurtransferase (3-MST), was reduced in liver but not other tissues. Aortic endothelial cell CSE was also reduced in high fat fed animals as determined immunohistochemically. Plasma H$_2$S concentration was not changed in these animals. No evidence of lipid deposition was apparent in aortae from high fat fed animals and plasma serum amyloid A (SAA) and C-reactive protein (CRP) were unchanged suggesting lack of frank atherosclerotic disease. Plasma IL-6, IL12p40 and G-CSF levels were increased by high fat feeding whilst other cytokines including IL-1$\alpha$, IL-1b and TNF-$\alpha$ were not altered. These results suggest that deficiency of tissue CSE and H$_2$S contributes to the low grade inflammation which occurs when mice are fed a high fat diet.
Introduction

Hydrogen sulfide (H\textsubscript{2}S), synthesised naturally by the enzymes, cystathionine \( \gamma \) lyase (CSE), cystathionine \( \beta \) synthetase (CBS) and 3-mercaptoppyruvate sulfurtransferase (3-MST), is increasingly being considered as an important regulator of a number of physiological and pathological processes (1,2). In particular, H\textsubscript{2}S exhibits a number of biological effects of relevance to inflammation. These include dilation of blood vessels, regulation of leukocyte adhesion and apoptosis and effects on pain sensation. However, despite extensive study, the precise role of H\textsubscript{2}S in inflammation is still not clear with both pro- and anti-inflammatory effects described in the literature (3). Moreover, H\textsubscript{2}S has recently been proposed to accelerate the resolution of an established inflammatory response (4) thereby adding a further dimension to the potential significance of this gas in inflammation.

H\textsubscript{2}S, and its donor drugs, are increasingly being considered as potential therapeutic agents for a number of clinical conditions including inflammatory disease (5,6). It is therefore important to understand the factors which determine the overall effect of this gas in inflammatory diseases. One such factor is likely to be the local concentration of H\textsubscript{2}S at the inflamed site. Sulfide salts (e.g. NaHS), which release H\textsubscript{2}S rapidly over a period of seconds to minutes, are usually pro-inflammatory in animal models of diseases (e.g. 7,8) whilst other donors such as GYY4137 (9) and ATB-346 (H\textsubscript{2}S-releasing-naproxen, 10) which release H\textsubscript{2}S slowly, over a period of hours, are generally anti-inflammatory in vivo.

Apart from the H\textsubscript{2}S concentration at the inflamed site, another factor which may play a part in determining the involvement of this gas in inflammation, is the duration and perhaps also the intensity of the inflammatory insult. To date, these
aspects has been largely ignored, since most researchers have studied the role of H\textsubscript{2}S in acute and/or severe inflammatory conditions in animals which persist either for a few hours (e.g. LPS-induced endotoxic shock, algogen-induced hindpaw swelling) or for a few days (e.g. colitis, arthritis). In these cases, the inflammatory state generated is both pronounced and functionally debilitating. To the best of our knowledge, there have been no attempts to assess whether H\textsubscript{2}S metabolism is altered in more long-lasting animal models of ‘low grade’ inflammation. Consequently, we have now examined the effect on tissue H\textsubscript{2}S biosynthesis and synthesising enzymes as well as on markers of inflammation and atherosclerosis of the long-term, low grade inflammation which follows administration of a high fat diet to mice.

**Materials and Methods**

(i) **Ethics statement**

All animal experiments were approved by the IACUC of the National University of Singapore (permit number 011/11).

(ii) **Animals and diet**

Mice (C57/Bl6, male, 23-25 g) were maintained in the Comparative Medicine department and fed a high fat (16% fat, 12.5% cholesterol and 5% sodium cholic acid) or normal diet (Research Diets Inc., USA) for up to 16 weeks. At the end of 8, 12 or 16 weeks, groups of animals were anaesthetised with a mixture of ketamine
(75mg/kg, i.p.) and medetomidine (1 mg/kg, i.p.) and blood obtained by cardiac puncture, anticoagulated with heparin (100 U/ml) and centrifuged (5000 r.p.m., 10 min, 4°C) to prepare plasma which was then stored at -80°C. Livers, kidneys and lungs were removed and immediately snap frozen in liquid nitrogen prior to biochemical analyses. Aortae were also removed and placed 4% v/v paraformaldehyde in phosphate buffer for subsequent histology.

(iii) Measurement of tissue H$_2$S synthesizing enzyme activity

Tissue H$_2$S synthesizing enzyme activity was determined as previously described (8). Briefly, lung, kidney and liver were homogenized (1:30 v/v) in ice-cold potassium phosphate buffer (100 mM, pH 7.4) (8). The assay mixture (500 µl) contained tissue homogenate (430 µl), L-cysteine (10 mM; 20 µl), pyridoxal 5’-phosphate (2 mM; 20 µl) (Sigma-Aldrich Ltd.), and saline (30 µl). For the detection of H$_2$S synthesizing activity by 3-MST, all reagents in the assay mixture remained the same, except that L-cysteine was replaced with 3-mercaptopyruvate (3MP) (0.1mM, 40µL) and pyridoxal 5’-phosphate was omitted. Thereafter, incubation was carried out in parafilm-sealed microcentrifuge tubes. After incubation (37°C, 30 min), zinc acetate (1% w/v, 250 µl) was injected to trap H$_2$S followed by trichloroacetic acid (10% w/v, 250 µl) to stop the reaction. Subsequently, N,N-dimethyl-p-phenylenediamine sulfate (20 mM; 133 µl) in 7.2M HCl was added followed immediately by FeCl$_3$ (30 µM; 133 µl) in 1.2M HCl, and absorbance (670 nm) of samples (300 µl) was read using a 96-well microplate reader (Tecan Systems Inc., USA). All standards and samples were assayed in duplicate. H$_2$S concentration of each sample was calculated against a
calibration curve of NaHS (3.125–250 µM) and results are expressed as nanomoles H$_2$S formed per milligram soluble protein using the Bradford assay (Bio-Rad).

(iv) Measurement of plasma H$_2$S concentration

Plasma H$_2$S was determined by a high-performance liquid chromatography (HPLC) method as described elsewhere (11). Briefly, mouse plasma (15 µl) and NaHS standards were derivatised (30 min, room temperature) with the fluorescent probe monobromobimane (MBB, 2 mM) in the dark. Thereafter, a standard curve of NaHS (0.018-1.5 µM) was prepared from the derivatised NaHS stock solution. HPLC analysis of derivatized plasma or NaHS solution was carried out on a C18 column using an Agilent 1100 Series HPLC System (Santa Clara, California, USA) with mobile phases comprising of 10% v/v methanol and 0.25% v/v acetic acid and 90% v/v methanol and 0.25% v/v acetic acid. Excitation and emission wavelengths were 385 nm and 475 nm respectively. Derivatised H$_2$S has a retention time in this system of 24.4±0.01 min (n=3).

(v) Western blotting for H$_2$S synthesizing enzymes

Lung, kidney and liver were homogenized (1:12.5 w/v) in lysis buffer comprising EDTA (5mM) containing protease and phosphatase inhibitors (Halt™ Protease Inhibitor Cocktail and Halt™ Phosphatase Inhibitor Cocktail) and 1% v/v Triton-X 100 in phosphate-buffered saline on ice, centrifuged (16,000 g, 10 min, 4°C) and homogenates collected. Protein concentration was quantified using the Bradford assay (Bio-Rad, California, USA). Samples were resolved on 12% SDS-PAGE and
transferred onto nitrocellulose membranes (Bio-Rad Ltd., California, USA) prior to incubation (4°C) overnight with blocking buffer (phosphate buffered saline, PBS, containing 5% v/v skimmed milk and 0.1% v/v Tween-20). Membranes were thereafter incubated (2 h, room temperature) with primary antibodies directed to CBS, CSE (Abcam Ltd., USA) or MST or actin (Sigma-Aldrich Ltd, USA), washed three times with PBS containing 0.1% v/v Tween-20 and incubated (1 h, room temperature) with secondary antibodies (goat anti-mouse IgG and goat anti-rabbit IgG, Thermo Scientific Pierce Ltd., Rockford, USA). The immunoreactive bands were visualized using chemiluminescent reagent (Merck Millipore Ltd., USA) and exposed to X-ray film. Resulting blots were scanned and quantified using ImageJ software.

(vi) Plasma cytokine and chemokine determination

A range of cytokines and chemokines were assayed in mouse plasma using a Bio-Plex Pro™ Mouse Cytokine 23-plex Assay (Bio-Rad, California, USA) according to the manufacturer’s instructions. Fluorescence was measured using the Luminex 100 system and results analyzed using Bio-plex Manager™ software (Bio-Rad, California, USA).

(vii) Measurement of plasma SAA and CRP

Plasma serum amyloid A (SAA) and C-reactive protein (CRP) levels were measured using ELISA kits (USCN Life Science Inc., Houston, USA and GenWay Biotech Inc., San Diego, USA) respectively, according to the manufacturer’s instructions.
(viii) Histology and immunohistochemistry

Tissue sections were fixed with 4% v/v paraformaldehyde in phosphate buffer at 4°C, washed with PBS and blocked with 5% v/v normal rabbit or mouse serum for 1 h at room temperature. Sections were then incubated overnight with the following primary antibodies: rabbit anti-CBS (1:200; Abcam, USA), rabbit anti-3-MST (1:200; Abcam, USA) and mouse anti-CSE (1:250; Abcam, USA) in PBS containing 0.1% v/v Triton X-100 (PBS-TX). Sections were washed in PBS and incubated in anti-rabbit IgG conjugated to FITC (1:200; Sigma) or anti-mouse IgG conjugated to Cy3 (1:200, Sigma) both diluted in PBS-TX for 1 h at room temperature and then counterstained with 4',6-diamidino-2-phenylindole (DAPI). Control sections were incubated as described above but without the primary antibodies and showed no significant staining. Photo-images were captured in a fluorescence microscope equipped with a digital camera (Olympus BX51, Olympus, Japan). For assessment of lipid staining, tissue sections were fixed with 4% v/v paraformaldehyde and washed briefly in water. Sections were then rinsed with 60% v/v isopropanol and stained (15 min) with Oil Red O solution (Sigma).

(ix) Statistics

Data is expressed as mean ± s.e. mean with the number of observations shown in parenthesis. Analysis was by Student's t-test and statistical significance of the difference between means was set at P<0.05.

Results
(i) Effect of a high fat diet on plasma H$_2$S concentration and tissue H$_2$S synthesising activity and CSE, CBS and 3-MST expression

Plasma H$_2$S concentration in control animals prior to administration of a high fat diet was 315.9 ± 10.0 nM (n=3) as determined by HPLC. No significant change in plasma H$_2$S concentration was detected in either control or fat fed animals at 8, 12 or 16 weeks (Figure 1).

The expression of individual H$_2$S synthesising enzymes in liver, kidney and lung from control and high fat fed animals was first determined by Western blotting. The effect of a high fat diet on expression of these enzymes was tissue-dependent. For example, both CSE and 3-MST were down regulated in liver after 8, 12 and 16 weeks of high fat treatment. In contrast, upregulation of CBS, perhaps compensatory in nature, was apparent in liver at 8 and 16 (but not 12) weeks (Figure 2A,D). Interestingly, expression of CBS in kidney was also upregulated at 8 and 16 (but not 12) weeks although in this tissue no significant effect on the expression of either CSE or 3-MST could be detected (Figure 2B,D). Like liver, lung CSE expression was also reduced at 12 and 16 weeks of feeding a high fat diet but, unlike liver, no change in the expression of either CBS or 3-MST was apparent in this tissue (Figure 2C,D).

Since significant expression of H$_2$S synthesising enzymes was downregulated in liver (CSE, 3-MST) and lung (CSE) in mice fed a high fat diet further experiments were conducted to monitor H$_2$S synthesis ex vivo in these tissues using, as substrate, either cysteine (to monitor CSE/CBS) or 3-mercaptopropionate (to monitor 3-MST) enzyme activity. Biosynthesis of H$_2$S from cysteine in the presence of
pyridoxal 5’ phosphate as cofactor was significantly (P<0.05) reduced in both liver
and lung from mice fed a high fat diet. Interpretation of these biosynthesis data may
be complicated by the augmented expression of CBS in livers of animals fed a high
fat diet which may partly compensate, in terms of H₂S generation, for the CSE
deficiency. 3-MST activity, determined specifically as conversion of 3-
mercaptopyruvate to H₂S, was also reduced in livers removed from mice fed a high
fat diet for 8, 12 and 16 weeks (Figure 3C).

(iii) Effect of a high fat diet on vascular CSE localisation

CSE was detected in endothelial, but not underlying vascular smooth muscle, cells in
aortic sections prepared from mice fed a control diet for 16 weeks (arrows in Figure
4). In contrast, endothelial cell CSE was greatly reduced or absent in the aortic
endothelial cell layer from mice fed a high fat diet for the same period (Figure 4B).
Neither CBS nor 3-MST were detectable in aorta from either control or high fat fed
animals at 16 weeks (data not shown).

(iv) Effect of a high fat diet on plasma cytokines and chemokines

No significant difference in plasma concentration of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-9,
IL-10, IL-12p70, IL-13, IL-17, eotaxin, GM-CSF, IFN-γ, CP-1, RANTES or TNF-α was
apparent between control animals and animals fed a high fat diet for 8, 12 or 16
weeks (data not shown). Feeding a high fat diet for either 12 or 16 weeks
significantly (P<0.05) increased plasma levels of IL-6, G-CSF and KC with no
change seen at 8 weeks (Figure 5A-D). In contrast, plasma IL12p40 was increased
in high fat fed mice at all time points tested. Plasma MIP-1b was also elevated at 12 and 16 weeks but reduced at 8 weeks (Figure 5E). The plasma concentration of IL-5 was increased at 16 weeks but not at other time points (Figure 5F).

(v) Effect of a high fat diet on biochemical and histological markers of atherosclerosis

Plasma concentration of SAA and CRP were not changed in mice fed a high fat diet (SAA: normal 16 weeks 3.71 ± 0.51 µg/ml; high fat 16 weeks 4.14 ± 0.94 µg/ml; CRP: normal 16 weeks 5.11 ± 0.20 ng/ml; high fat 16 weeks 4.65 ± 0.20 ng/ml, all n=6, P>0.05). Oil red O stained sections of aorta from both control and 16 week high fat fed mice showed essentially normal histology as characterised by a thin intima lined by endothelium and a thick media with no evidence of fatty streaks (Figure 6A,B).

Discussion

It has long been known that excess fat in the diet is a risk factor for the development of atherosclerosis in man (12) and that restriction of fat intake is beneficial in some cardiovascular diseases (13). Whilst rodents are generally resistant to fat-induced atherosclerosis (14) there is growing evidence that feeding mice a high fat diet causes a low grade systemic inflammation which persists for several months and contributes to both atherosclerosis and metabolic diseases such as obesity (15-18). In man, atherosclerosis is considered to be an inflammatory disease (19) and lipid-lowering statins, which are the mainstay of its treatment, also exhibit significant anti-
inflammatory activity (20). Lack of H$_2$S formation has previously been proposed to contribute to the development of atherosclerosis in animals (21). Moreover, as noted previously, H$_2$S plays complex roles in both acute and chronic inflammatory disease states (3). Whether H$_2$S has any role to play in high fat-induced chronic inflammation and whether changes in H$_2$S synthesis or synthesising enzymes precede the development of atherosclerotic disease is not known.

We show here that feeding mice a high fat diet did not affect plasma H$_2$S concentration but did alter the expression of tissue H$_2$S synthesising enzymes. This effect was dependent on the length of time of high fat feeding and on the tissue studied. Reduced CSE and 3-MST expression and H$_2$S biosynthesis from L-cysteine (catalysed by CSE or CBS) or 3-mercaptopyruvate (catalysed by 3-MST) occurred in the livers of high fat fed mice and was more apparent towards the end of the feeding period i.e. 12-16 weeks. In separate experiments, aortic endothelial CSE expression assessed immunohistochemically was also reduced after 16 weeks of a high fat diet. We conclude that high fat feeding in these animals resulted in a slowly developing decline in tissue H$_2$S biosynthesis and CSE expression. Interestingly, using a specific and sensitive HPLC assay we were not able to detect a difference in plasma H$_2$S concentration between controls and high fat fed animals at any time point. This may not be surprising bearing in mind the chemically and metabolically evanescent nature of this gas. As such, we conclude that plasma levels of H$_2$S do not reflect changes in enzyme expression/activity at least in tissues from high fat fed animals.

The deficiency in H$_2$S biosynthesis identified in the current study precedes the development of frank atherosclerosis in these animals. This was shown histologically in high fat fed animals, by the lack of demonstrable aortic lipid deposition and also biochemically, by the finding of unchanged plasma concentrations of SAA and CRP.
both of which are characteristic early markers of atherosclerosis (22,23). In addition, the plasma concentration of a number of cytokines including IL-1α, IL-1β, IL-2, IFN-γ and TNF-α all of which are known to play key roles in initiating and/or sustaining full blown atherosclerosis (24) was not changed even after 16 weeks of high fat feeding. This implies the absence of overt systemic inflammatory disease in these animals. However, the plasma concentration of a limited number of plasma cytokines and chemokines was increased in animals fed a high fat diet. These include IL-5, IL-6, IL-12p40, MIP-1β and G-CSF all which were elevated in plasma at one or more time points. Thus, some (or all) of these cytokines might be considered as candidate biomarkers for the low grade inflammation which precedes the more classical inflammatory disease associated with atherosclerosis. Interestingly, IL-6 and IL-12p40 were the most consistently increased in plasma from high fat fed mice. Of these, IL-6 has both pro- and anti-inflammatory activities with many diverse effects on monocytes and endothelial cells (25) and has been widely suggested to be a marker for cardiovascular disease in man (26) whilst IL-12p40 is a subunit not only of bioactive IL-12p70 but also, in conjunction with IL-23p19, of bioactive IL-23. Both IL-12 and IL-23 are important for the regulation of cell-mediated immune responses and T helper 1 (Th1)-type inflammatory reactions (27) and have been identified as possible targets for novel therapeutics to treat inflammatory conditions such as colitis (28). Intriguingly, IL-12 p40 mRNA is also abundant in human atherosclerotic plaque (29).

In conclusion, these are the first data which show a time-dependent deficit of CSE and, in some tissues also 3-MST as well as H₂S synthesis from added precursors, in tissues from mice fed a high fat diet. This observation was coupled with evidence of low grade systemic inflammation in these animals as revealed by
changes in the plasma cytokine profile. Since changes in tissue H₂S synthesis and plasma cytokine concentrations occurred at more or less the same time points after starting a high fat diet it is not clear if the deficiency of H₂S detected in these animals promotes or retards the low grade inflammation associated with fat feeding. The possibility that deficient H₂S biosynthesis/CSE expression in tissues from fat fed animals predicts the subsequent occurrence or severity of atherosclerosis warrants further study.
References


**Figure legends**

**Figure 1**

Plasma H$_2$S concentration in control (open columns) and high fat fed (closed columns) mice at 8, 12 and 16 weeks. Results show H$_2$S concentration (nM) and are mean ± s.e. mean, n=3-7.

**Figure 2**

Expression of CBS, CSE and 3-MST by Western blotting in liver (A), kidney (B) and lung (C) from control (open columns) and high fat fed (closed columns) mice at 8, 12 and 16 weeks. Results show expression of each protein (c.f. actin) and are mean ± s.e. mean, n=3, *P<0.05. Representative blots are shown in (D).

**Figure 3**

Biosynthesis of H$_2$S from added cysteine in liver (A) and lung (B) and from added 3-mercaptopryuvate in liver (C) from control (open columns) and high fat fed (closed columns) mice at 8, 12 and 16 weeks. Results show H$_2$S synthesis from added precursor expressed nmol/mg protein and are mean ± s.e. mean, n=10-14, *P<0.05.

**Figure 4**
Representative photographs showing immunohistochemical identification of CSE in transverse sections of aorta from control (A) and high fat fed mice (B) after 16 weeks. Arrows highlight areas of brown CSE staining. Scale shows dimensions (20 μm).

**Figure 5**
Concentration of a range of (A) IL-6, (B) IL-12p40, (C) G-CSF, (D) KC (E) MIP-1β and (F) IL-5 in plasma from in control (open columns) and high fat fed (closed columns) mice at 8, 12 and 16 weeks. Results show concentration of cytokine/chemokine in pg/ml and are mean ± s.e. mean, n=6-7, *P<0.05.

**Figure 6**
Representative photographs showing Oil red O staining for lipid in aorta from control (A) and high fat fed (B) mice at 16 weeks. Scale shows dimensions (20 μm).