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Dietary homocysteine promotes atherosclerosis in apoE-deficient mice by inducing scavenger receptors expression

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Abstract

Elevated plasma homocysteine (Hcy) levels have been recognized as an independent risk factor for atherosclerosis leading to cardiovascular diseases. However, the mechanisms contributing to atherosclerosis have not been delineated. Since, scavenger receptors mediated uptake of oxidized-LDL (oxLDL) by macrophages resulting in foam cell formation is an early event in atherosclerosis, we hypothesized that atherogenic effects of Hcy may be mediated via regulating expression of scavenger receptor(s). We have tested this hypothesis using apoE−/− female mice fed normal rodent chow (NC) diet or NC supplemented with Hcy in drinking water (9 g/L). Hcy-fed mice showed increased fatty streak lesions in aortic sinus/root compared to NC group without alterations in plasma lipid profiles. Similar findings were observed in the *enface* analysis of the descending aorta. To determine the molecular mechanisms underlying Hcy-mediated progression of fatty streak lesions, expression of scavenger receptors such as CD36 and lectin-like oxidized LDL binding protein-1 (LOX-1) in the aortic lesions were analyzed. Interestingly, Hcy-fed mice had increased immuno-positive staining for CD36 and LOX-1 in the atherosclerotic lesions compared to NC-fed mice. In vitro analyses showed neither Hcy nor HcyLDL directly affect the expression of CD36 and LOX-1 on mouse macrophages. However, Hcy supplementation in apoE−/− mice resulted in elevated oxLDL levels in plasma. Since oxLDL has been shown to upregulate the expression of CD36 and LOX-1, these findings suggest that Hcy may exert its atherogenic effect in part by elevating the levels of oxLDL. Interestingly, interaction of monocytes with Hcy-activated endothelial cells resulted in upregulation of CD36 expression on monocytes, suggesting a possible mechanism by which Hcy may upregulate CD36 expression at the lesion site. Further, these findings suggest a novel mechanism by which Hcy may promote atherogenesis.

Keywords: Atherosclerosis; Homocysteine; Scavenger receptors; CD36; LOX-1; Foam cells

1. Introduction

Several risk factors including hypercholesterolemia, diabetes and tobacco use have been implicated in the onset of atherosclerotic lesions [1]. In addition to these established risk factors, epidemiological studies have indicated that elevated plasma levels of Hcy may be an independent risk factor for atherosclerosis and thrombosis [2,3]. Hcy is a non-protein thiol containing amino acid, which is produced in the cell as an intermediary in methionine metabolism [4]. Hcy

Abbreviations: ApoB, apolipoprotein B; apoE−/−, apolipoprotein E knockout; CBS, cystathionine β-synthase; DAB, 3,3′-diaminobenzidine; Hcy, homocysteine; HcyBSA, Hcy-modified BSA; HcyLDL, Hcy-modified LDL; LOX-1, lectin-like oxidized LDL binding protein-1; NC, normal rodent chow; oxLDL, oxidized LDL; SR-A, scavenger receptor-AI/II.

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is metabolized to cystathione by cystathionine β-synthase (CBS) via the transsulfuration pathway requiring vitamin B6 [4]. Alternatively, Hcy is remethylated to methionine via methionine synthase requiring vitamin B12 as cofactor and 5′-methyltetrahydrofolate [4]. Consequently, deficiency in enzymes and vitamin B cofactors involved in Hcy metabolism can result in homocysteinemia.

Diet-induced homocysteinemia either by feeding high methionine with low B vitamins [5,6] or Hcy in the drinking water [7,8] have been shown to accelerate atherosclerotic lesion development in apoE−/− mice. Accelerated atherosclerosis has also been reported using apoE−/− CBS−/− double knockout mice-fed normal chow diet [9]. Hofmann et al. [5] have suggested that accelerated lesion was due to redox stress of the vasculature as evidenced by elevated levels of NFκB and NFκB-regulated genes such as vascular cell adhesion molecule-1 and receptor for advanced glycation end products. In vitro studies using human monocytes treated with Hcy (100 μmol/L) resulted in increased NADPH oxidase dependent reactive oxygen species generation [10]. Moreover, in a recent report Dai et al. [8] have shown that apoE−/− mice supplemented with Hcy in drinking water have increased expression of NADPH oxidase ($p47^{phox}$) in the aortic lesion. In all these studies Hcy-induced acceleration of atherosclerosis was determined by Oil Red O staining for fatty streak lesions. However, the mechanism(s) by which Hcy contributes to the foam cell formation leading to fatty streak lesions, an early event in atherogenesis is unknown.

Generation of oxLDL and its uptake by macrophages leads to lipid-laden foam cell formation, which plays a central role in the initiation and progression of atherogenesis [11]. The proatherogenic function of oxLDL is mediated by scavenger receptors including scavenger receptor AI/II (hereafter referred as SR-A), CD36, and LOX-1 [12,13]. Among these SR-A and CD36, expressed on macrophages have been implicated in the uptake oxLDL resulting in the formation of foam cells in vitro [14–16] and progression of atherosclerosis in vivo [15,17,18]. Due to the central role of scavenger receptors in foam cell formation leading to fatty streak lesions, we hypothesize that Hcy may exert its atherogenic effect by regulating the expression of scavenger receptors. The aim of this study was to determine the role of scavenger receptors in Hcy-accelerated atherosclerotic lesion formation in apoE−/− mouse model.

2. Materials and methods

2.1. Animals and diet

Female apoE−/− mice [19] obtained from Jackson Laboratories were housed in micro isolator cages. The mice (4-weeks old) were assigned to a control group fed a standard rodent diet (Harlan Teklad # 8640) and were designated as normal chow (NC). A second group were fed the same diet and supplemented with 9 g/L DL-Hcy (Sigma) in drinking water and designated as Hcy-fed group. Mice were allowed free access to food and water. Water bottles without and with Hcy were changed every third day and water intake was recorded throughout the study period. All the animals were weighed and observed for any physical changes on a weekly basis. Mice were euthanized after 12 weeks on diet, blood, heart, and aorta were collected for further analyses. The Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences approved the animal protocol for this study.

2.2. Plasma lipids and Hcy

Non-fasting blood samples were obtained and plasma total cholesterol, triglyceride, and HDL-cholesterol were determined by a microplate enzymatic assay [20] using reagents from Synermed International Inc. LDL-cholesterol was calculated using Friedewald's formula (LDL-cholesterol = [total cholesterol − HDL-cholesterol-(triglycerides/5)]. Plasma total Hcy levels was determined by HPLC method described in detail in an earlier publication [21].

2.2.1. Atherosclerotic lesion analyses

The heart and aorta up to the iliac bifurcation were removed and analyzed for atherosclerotic lesions in aortic root and descending aorta (*enface* analysis). After removing peripheral fat, the heart was fixed overnight in PBS/3% paraformaldehyde/20% sucrose buffer, and then embedded in optimal cutting temperature cryosectioning medium (Tissue Tek). Atherosclerotic fatty streak lesions in the aortic root were analyzed by Oil Red O staining (Sigma) and counterstained with hematoxylin according to the method of Paigen et al. [22]. Atherosclerotic lesions were captured as digital images by AxioCAM-HRc color camera attached to an inverted Carl Zeiss microscope and total mean lesion area was quantified using AxioVision image analysis software (Carl Zeiss). Severity of lesions in the aortic root sections was characterized by Movat's pentachrome staining (Electron Microscopy Sciences) as previously described [19]. A veterinary pathologist analyzed the Oil Red O and Movat's pentachrome stained sections in a blinded manner and determined the extent of atherosclerotic lesion severity as per the American Heart Association's guidelines [23].

2.2.2. Enface analysis

The descending aorta was fixed in buffered formalin, opened longitudinally and stained with Sudan IV as previously described [22]. Atherosclerotic lesions were captured as digital images using an Carl Zeiss stereo microscope with a AxioCAM-MRc color camera and mean total lesion area was quantified using AxioVision image analysis software as previously described [22].