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Atherogenesis: hyperhomocysteinemia interactions with LDL, macrophage function, paraoxonase 1, and exercise

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Abstract

Despite great strides in understanding the atherogenesis process, the mechanisms are not entirely known. In addition to diet, smoke, genetic predisposition, and hypertension, hyperhomocysteinemia (HHcy), an accumulation of the noncoding sulfur-containing amino acid homocysteine (Hcy), is a significant contributor to atherogenesis. Although exercise decreases HHcy and increases longevity, the complete mechanism is unclear. In light of recent evidence, in this review we focus on the effects of HHcy on macrophage function, differentiation, and polarization. Though there is need for further evidence, it is most likely that HHcy-mediated alterations in macrophage function are important contributors to atherogenesis, and HHcy-countering strategies, such as nutrition and exercise, should be included in the combinatorial regimens for effective prevention and regression of atherosclerotic plaques. Therefore, we also included a discussion on the effects of exercise on the HHcy-mediated atherogenic process.

Keywords

hyperhomocysteinemia; atherosclerosis; macrophage; oxidized LDL; PON1; exercise

Introduction

Atherosclerosis is an arterial pathology involving progressive accumulation of cholesterol deposits and eventual buildup of plaque inside the arterial wall. Such plaque buildup results in narrowing of the arterial lumen with a bulge in the arterial wall, thereby reducing blood supply to the end organs. Rupture of vulnerable plaques contributes to thrombus formation, which critically obstructs the blood flow to the end organs, thereby leading to many cardiovascular diseases: stroke, acute myocardial infarction, and peripheral vascular disease—all of which are leading causes of mortality and morbidity globally.¹ Recently, there have been seminal advances in prevention, including computed tomography (CT) scans, angiograms, and magnetic resonance imaging (MRI) to gauge the vulnerable plaques, and in treatment, such as reversal of elevated lipids directly related to plaque buildup and instability, reinforcement of blood supply by stenting, and thrombectomy. Despite such

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Conflicts of interest

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advances, atherosclerosis remains the leading cause of death.^{2,3} The complexity and slow progression of atherosclerosis, along with our incomplete understanding, renders these combined efforts relatively ineffective. This review examines the key aspects of the disease: lipids, macrophages, oxidative stress, and physical activity, as well as the independent cardiovascular risk factor hyperhomocysteinemia (HHcy), and how they relate to each other in either initiation (endothelial dysfunction) or progression of atherosclerosis (plaque formation and rupture).

Background

Atherosclerosis has historically been viewed as the collection of cholesterol and thrombi in the arterial wall.⁴ However, in 1858, Rudolf Virchow recognized it as more of an active inflammatory process of tissue reaction than a simple process of fat accumulation. This pathology can form at an early age in humans, and actively progresses to varying degrees of severity.⁵ Although passive lipid deposition has been the main focus of studies of atherosclerosis, and the involvement of macrophages in inflammatory response has been known for over a hundred years, the complete mechanisms underlying macrophage-involved lipid processing and progression of atherosclerosis remains unclear. To date, the literature indicates that atherosclerosis is the result of non-resolving interactions of heightened oxidative stress, inflammation, immune response, lipid deposition, and genetic predisposition.^{6,7} We have summarized the critical players and steps of atherogenesis in Figure 1.

The initial insult to the endothelial vascular layer that initiates atherosclerosis remains unknown. It is believed that native antigens in the circulation, such as low density lipoprotein (LDL) and its oxidized form, oxidized LDL (OxLDL), may accumulate in the arterial intima and activate adaptive immunity, leading to the cascade of events resulting in atherosclerosis.⁸ This appears as early as fetal stages of development⁹ and forms what is called the “fatty streak.”¹⁰ Other factors, such as hypertension,^{11,12} smoking,^{13–15} and HHcy,¹⁶ that cause endothelial cell injury and consequent endothelial dysfunction—early steps of atherosclerosis—have also been reported. Interestingly, there are certain commonalities between these risk factors in causation of endothelial dysfunction: elevated oxidative stress and reduced nitric oxide (NO) bioavailability. There are also strong genetic components involved in the regulation of ROS, homocysteine (Hcy) levels, and atherogenesis.^{17–22} All these factors, along with the quality of nutrition²³ and environment (air pollutants)²⁴ influence, either directly or indirectly, the conversion of LDL to OxLDL, a crucial process in atherogenesis.

Endothelial cells and smooth muscle cells (SMCs), critical components of the arterial wall, respond to OxLDL and produce interleukins and cytokines that enhance the adhesiveness of endothelial cells and eventually attract different inflammatory cells.^{10,25,26} VCAM-1, the cell-adhesive protein induced by OxLDL, draws monocytes and T lymphocytes to the endothelium in early atherogenesis.^{4,27,28} Additional signals, such as monocyte chemoattractant protein-1 (MCP-1),²⁹ P-selectin, ICAM-1,^{10,30} and macrophage colony-stimulating factor (M-CSF),^{31,32} also cause circulating monocytes to enter the intima layer of the arterial wall and plaque. Monocytes will differentiate into macrophages, which engulf

OxLDL through scavenger receptors,^{4,10} such as CD68,^{33–35} SR-A,³⁶ CD36,³⁷ LOX-1,³⁸ and others.^{39–41} Thus, monocytes and macrophages play critical roles in disease progression,^{42–44} resulting in further recruitment and differentiation of macrophages.⁴⁵ Normally, macrophages expel cholesterol through the PPAR γ –liver X receptor (LXR)–ATP-binding cassette A1 (ABCA1) pathway and thus are not prone to becoming foam cells.^{38,46–51} However, this is not the case with the oxidized cholesterol OxLDL. Unable to process OxLDL, lipid-laden macrophages become foam cells, which are the hallmarks of atherosclerotic lesions and vulnerability.^{4,52,53} Progression from fatty streak to protruding lesion occurs when the SMCs migrate from the medial cell layer of the artery to the intimal layer in response to the ongoing inflammation and proliferate. The SMCs,⁵⁴ along with infiltrated macrophages, also take up OxLDL and synthesize extracellular matrix metalloproteases (MMPs), whose substrates include various components of the plaque and the surrounding tissue matrix.⁵⁵ MMP production leads to disruption in the collagen-to-elastin ratio and buildup of fibrous cap,¹⁰ which may temporarily prevent plaque rupture.⁵⁶ In fact, 11 of the 28 known human MMPs have been clinically associated with atherosclerosis and atherothrombosis.⁵⁵

Through yet-to-be identified mechanisms, the plaques further progress into the terminal stage: plaque rupture. After a certain extent of lipid uptake and foam cell formation, macrophages and SMCs⁵⁷ undergo apoptosis, generating necrotic cores in the plaques, a sign of potential rupture.^{4,58–60} Plaque rupture is most likely to occur along the shoulder regions of the plaques, especially those located at the bifurcation sites of arteries.⁶⁰ Consistent with this phenomenon, M-CSF localizes to the shoulder regions of plaques.⁵⁸ Dysfunctional clearing of debris and apoptotic cells within the necrotic core further exacerbates plaque formation in the arterial intima.⁶¹ Although further evidence is necessary for substantiation, it is possible that macrophages do not initiate atherogenesis, as the process starts with endothelial injury, but they are critical components in plaque formation and rupture (atherothrombosis).

Role of homocysteine

An elevated level of Hcy, HHcy is a risk factor for atherosclerosis and morbidity related to atherothrombosis.^{62–64} HHcy is also an independent risk factor for cardiovascular disease (CVD), specifically hypertension, as it increases the ability of vascular smooth muscle cells to react to angiotensin II (AII).^{65,66}

Hcy is a non-protein coding, sulfhydryl-containing amino acid.⁶⁷ It is formed during metabolism of methionine and is converted to cysteine or methionine by various enzymes and vitamin B cofactors.^{68,69} The fate of Hcy metabolism depends on the relative strength of the two pathways: (1) irreversible transsulfuration to cysteine and (2) remethylation to methionine.⁷⁰ Methionine and ATP are catalyzed to S-adenosylmethionine (SAM) by methionine adenosyltransferase (MAT). The product SAM is the substrate for SAM-dependent methyltransferases, which feed methyl groups to cellular methylation reactions (e.g., DNA, RNA, proteins, neurotransmitters), and produces S-adenosylhomocysteine, which will be converted to Hcy by S-adenosylhomocysteine hydrolase. The enzyme methionine synthase (MS) remethylates Hcy to methionine and links the folate pathways

with Hcy pathways. MS is one of two methionine synthases responsible for remethylation of Hcy to methionine, the other being betaine homocysteine methyltransferase (BHMT), whose expression is limited to the liver and kidneys, whereas the former is ubiquitously expressed.⁷¹ Alternatively, Hcy is metabolized to cystathionine by cystathionine beta synthase (CBS) and then to cysteine by cystathionine γ -lyase (CSE), both of which require vitamin B6 as a cofactor.

HHcy contributes to atherogenesis and CVD through several mechanisms (Fig. 2): (1) heightened oxidative stress—HHcy induces oxidative stress/ROS, either through induction of thrombin and consequent activation of PAR-4 and NADPH oxidase 1 or through oxidation of reactive sulfhydryl groups in the presence of molecular oxygen;^{66,67,72,73} (2) altered vascular responses and endothelial injury—Hcy induces hypertension via vasoconstriction through increased arterial stiffness⁶⁵ and hence is the risk factor for atherosclerosis; (3) enhanced propensity for plaque rupture—high levels of plasma Hcy correlate with atherosclerotic plaque rupture and morbidity, as with type 2 diabetes, another independent risk factor for cardiovascular disease, plaque rupture, and death;^{62,74–79} (4) inflammation and cell adhesion—HHcy upregulates the CD40/CD40L system and the number of cells and platelets expressing CD40/CD40L, and VCAM-1 expression is induced by CD40L in endothelial cells;⁸⁰ (5) by promoting vascular SMCs activation and enhancing their proliferation;⁸¹ and (6) potentially through macrophage activation and differentiation. Owing to space limitations, we have not discussed the role of HHcy in SMC proliferation. The macrophage-related HHcy effects will be discussed in the following sections.

Interestingly, however, populations with exceptionally low gene frequency for HHcy predisposition show a lack of correlation between lipoproteins and Hcy as risk factors for atherosclerosis.⁸² However, other reports suggest that certain genetic polymorphisms predispose to HHcy as well as enhanced risk for atherogenesis.^{19,20} It is possible that certain genetic backgrounds confer higher predisposition to HHcy-mediated atherogenesis while others do not. Factors such as ROS accumulation/generation capacity might have modifier effects. Polymorphisms of PON1 (an HDL-associated enzyme involved in countering HHcy toxicity) might also be considered as modifiers of HHcy-induced atherogenesis (discussed below). Taking these results together (Fig. 2), the notion that HHcy is an independent risk factor for atherogenesis has generous support in the literature, but more studies are needed to clarify its role as an independent risk factor versus an aggravator and to provide further mechanistic insights.

Role of oxidized LDL

As reviewed by Glass *et al.*¹⁰ and Witztum and Steinberg,⁸³ circulating cholesterol is bound to proteins. Very low-density lipoprotein (VLDL), rich in triglycerides, is synthesized in the liver and gradually loses triglycerides in the peripheral tissue, with a portion of VLDL metabolized to LDL and finally to high-density lipoprotein (HDL), which ends up transporting the majority of cholesterol. Although both HDL and LDL are essential for delivering cholesterol to the cells, excess levels of LDL are associated with increased risk for CVD and death, while excess HDL is associated with cardiovascular health. A feedback mechanism prevents excessive cholesterol accumulation in the macrophages by

downregulation of LDL receptor, which only binds the unmodified LDL.⁸⁴ Macrophages also expel excessive cholesterol via the PPAR γ -LXR-ABCA1 pathway.^{50,51} However, it is the OxLDL that potentially overrides all these preventive strategies of excessive cholesterol accumulation in the macrophages via its binding and internalization through scavenger receptors, causing rapid accumulation of cholesterol. Furthermore, OxLDL can also upregulate these scavenger receptors, leading to excessive cholesterol accumulation and the formation of foam cells. OxLDL also disrupts the ability of endothelial cells (ECs)⁸⁵ and adipocytes⁸⁶ to induce ABCA1 through inhibition of LXR function. For ECs, OxLDL induces ICAM-1, a monocyte chemoattractant protein, leading to recruitment of monocytes/macrophages, and initiates a vicious cycle of macrophage loading and the conversion of macrophages into foam cells, culminating into plaque formation.³⁰ In addition to its role in foam cell formation, OxLDL affects macrophages by reducing their motility, resulting in prolonged residency in the arterial intima.⁸⁷ Thus, OxLDL came to prominence as a causative factor for atherogenesis. Additionally, treatment with antioxidants in primate and nonprimate models reduces the development of atherosclerosis.^{10,88} Thus, lipoproteins and oxidative stress have been associated with atherosclerosis from an early point.

There are at least 11 different scavenger receptors for OxLDL internalization into macrophages, categorized into classes A, B, and D depending on their structure.^{89,90} Macrophages have most of these receptors, including CD36 (class B), CD68 (class D), CLA-1, SR-A (class A), and LOX-1 (class D), but only the first three are associated with an increase in foam cell formation,⁹¹ implying these are the main transporters. ApoE-deficient (ApoE^{-/-}) mice that are also deficient in scavenger receptors CD68,^{92,93} CD36,³⁷ or SR-A³⁶ exhibit significantly less lesion development than control ApoE^{-/-} mice. Interestingly, LDL can enhance the expression of all three receptors.⁹⁴ However, OxLDL only increases CD68 levels. Although the surface expression of CD36 and CD68 is limited in nonactivated monocytes, after activation (activated macrophages) CD68 could significantly contribute to the uptake of OxLDL.^{91,95,96} When a soluble form of CD68 was injected into a murine model, there was a reversal of atherosclerosis plaque size, less foam cell formation, and less leukocyte migration into the arterial intima.^{92,93}

Formation of OxLDL is proposed to occur when LDL is exposed to the oxidative products from the endothelial cells, macrophages, and SMCs in the subendothelial space. Although several factors, including HHcy, were shown to cause OxLDL formation from LDL, the exact mechanisms are unclear.⁸⁴ It was shown that metal ions (iron and copper), oxidative enzymes (lipoxygenase and myeloperoxidase) and superoxide radicals with or without NO presence can catalyze the formation of OxLDL. Moreover, air pollution was shown to be positively correlated with OxLDL presence.⁹⁷ Air pollution in general increases systemic oxidative stress and inflammation, which might catalyze the formation of OxLDL.⁹⁸ Interestingly, diabetes, kidney failure, and infectious diseases, which also increase oxidative stress and inflammation, were shown to enhance OxLDL and atherogenic risk.⁸⁴ In addition, the type of fat content in the diet also influences the susceptibility of LDL for oxidation. A diet rich in saturated fatty acids caused more susceptibility for OxLDL formation, while a diet rich in monounsaturated fatty acids caused resistance.⁹⁹ Moreover, supplementation of antioxidants and paraoxonases through diet have yielded reduced LDL oxidation.¹⁰⁰ In contrast, a diet rich in oxidized cholesterol accelerated atherosclerosis in the absence of LDL

receptor and apolipoprotein E.¹⁰¹ These findings further emphasized the role of oxidative stress in the formation of OxLDL and consequent atherogenesis.

A combination of high OxLDL levels and high Hcy levels has been shown to diminish coronary flow rates.¹⁰² Under certain conditions, Hcy has been shown to enhance metal-catalyzed oxidation of LDL, and the pH of the surroundings determines whether the metal-catalyzed oxidized product is cytotoxic to macrophages.^{103,104} Hcy-related increases in oxidized stress, such as increased iNOS and MPO production, may indirectly result in increases in OxLDL. Furthermore, dietary supplementation with B vitamins (folate, B6, and B12) not only reduced HHcy levels but also suppressed HHcy-induced atherosclerotic lesions.¹⁰⁵ Both OxLDL and Hcy increase platelet adhesion to endothelial cells, albeit via independent mechanisms.¹⁰⁶ These findings imply that an elevated OxLDL and HHcy combination could result in rapid development and progression of atherosclerotic lesions. More studies are necessary to clarify if HHcy also aggravates the OxLDL-induced effects on macrophages, such as induction of CD68 and CD36 scavenging receptors, and leads to foam cell development.

Role of macrophages

Macrophages are essential in inflammation resolution,⁶¹ and thus their presence in atherosclerotic plaques⁴ should not seem unusual. As discussed earlier, circulating monocytes migrate, adhere, and pass through the endothelial cell layer of arterial vessels and preferentially accumulate in the subendothelial layer. The significance of monocyte adhesion and uptake of lipoproteins is seen when there is an almost complete absence of atherosclerosis after M-CSF or P-selectin (a platelet- and endothelial-related macrophage adhesion molecule) knockouts are introduced into murine models of dyslipidemia.^{31,107–109} Additionally, mice deficient in LDL receptors with marked reduction in VCAM-1 exhibited less plaque formation than controls, which is also the case when the VCAM-1 interaction is blocked with integrins in the ApoE knockout model.^{110–112}

Macrophages have recently been parsed into at least two broad subtypes—M1 and M2—each of which plays specific roles in atherosclerosis^{4,38,59,113,114} The M1 macrophages are known as “classically activated” macrophages and are associated with inflammation, cell killing, and bacterial infections. The M2 macrophages are known as “alternatively activated” macrophages and include three different subtypes: (1) the M2a subtype is profibrotic and counters parasitic infections, (2) the M2b subtype is involved in immunity regulation, and (3) the M2c subtype promotes the anti-inflammatory response and tissue repair and remodeling.^{59,111} There are specific factors or cytokines that cause the polarization of a macrophage to become M1 or M2. The factors IL-4 and IL-10, secreted by T_H2 cells, cause a shift towards M2,^{61,115} while factors such as IFN γ are responsible for M1 polarization.¹⁰⁹ M1 macrophages express high levels of CD40, CD80, IL-6, IL-12, TNF- α , iNOS, and lower expression of IL-10;¹⁰⁹ while M2 macrophages express higher levels of CD163, CD206,¹¹⁶ arginase-1, IL-1 β , and IL-10.^{109,111} Induction of the M2 phenotype depends on elevation of scavenger receptors CD36 and SR-A1, which cause ER stress enhancement.^{117,118} Interestingly, the role of the specific OxLDL scavenger receptor CD68 has not been investigated in the causation of ER stress and M2 polarization. Also, the presence of M2

macrophages leads to more foam cell formation owing to the higher phagocytic nature and greater capacity to import OxLDL with the low capacity to efflux ingested cholesterol.³⁸ OxLDL further affects functions of M1¹¹⁹ and M2 macrophages, the latter by increasing phagocytic activity and decreasing cholesterol efflux by disrupting the LXR pathway.³⁸ Foam cell-prone M2 macrophages have a greater presence than proinflammatory M1 macrophages in the early atherosclerotic lesions; however, the ratio reverses as the lesion progresses.¹²⁰ M1 macrophages also preferentially allocate to shoulder regions of plaques, where rupture is more likely to occur.¹²¹ Adding to the complexity, it was found that in the human carotid atherosclerotic lesions there was greater accumulation of Cd68⁺ but non-M2 type macrophages at the lipid core.³⁸ Hence, it is yet to be clarified whether mere M2 to M1 switching is enough to control foam cell formation and atherogenesis, as proposed earlier.¹¹⁷ Failure to clear debris and OxLDL from plaque sites by macrophages (efferocytosis) leads to more cell accumulation and further necrosis, amplifying the necrotic core, destabilizing the plaque, and leading to rupture.^{61,122,123}

Potentially, HHcy may be a major cause for the dysfunction of macrophages that leads to the unfortunate snowball effect that is atherosclerosis. As macrophages are crucial in inflammation resolution, and their impairment in doing so results in necrosis and greater sclerotic growth, it is imperative to assess HHcy-mediated oxidative stress enhancement and ER stress effects on macrophage dysfunction. In this regard, it has been showed that an HHcy condition not only elevates plasma levels of inflammatory cytokines (TNF- α , IL-6, and MCP-1), but also enhances vessel wall monocyte accumulation and their differentiation, leading to accelerated atherosclerosis, and that antioxidants suppressed these processes.¹²⁴ Macrophages also produce H₂S, a novel modulator of inflammation and one of the end products of the transsulfuration pathway of Hcy metabolism that irreversibly removes Hcy from the system. It is formed via CBS or CSE, both pyridoxol-5'-phosphate (PLP)-dependent enzymes, and 3-mercaptopyruvate sulfurtransferase (3MST), a PLP-independent enzyme. During conditions of high plasma Hcy (HHcy), Hcy competes with cysteine for binding to CSE, and therefore might decrease H₂S production.⁶⁶ Besides the potential of lowering the anti-inflammatory H₂S (both in terms of plasma levels and production by macrophages), HHcy is also associated with increases in proinflammatory cytokines (and coincidentally M1 markers) such as TNF- α and IL-1 β .¹²⁵ Additionally, H₂S has several cytoprotective effects, including inhibiting myocardial injury caused by HHcy.^{125–128} Given that ER stress plays a crucial role in macrophage function and that both H₂S¹²⁹ and Hcy²² can modulate ER stress, it is interesting to study the effects of HHcy and a lack of H₂S on macrophage polarization. Importantly, the plasticity of macrophages (M1 to M2 and M2 to M1) is crucial for inhibition of plaque progression and for macrophage exit from the lesions at the appropriate time. In this regard, whether HHcy attenuates macrophage plasticity and enhances lesion progression needs to be determined to understand the specific nature of HHcy-induced macrophage dysfunction in the atherosclerotic lesions.

Apart from inflammatory cytokine secretion and foam cell formation, macrophages also produce MMPs during atherogenesis. HHcy affects the activity of several of these MMPs. Specifically, it activates MMP-2¹³⁰ and induces and enhances MMP-9 production via the ERK pathway (Ca²⁺ dependent);^{131,132} both of these are associated with CVD. MMP-2 and MMP-9 process similar substrates, and MMP-9 is specifically associated with systolic

hypertension and arterial stiffness,¹³³ unstable plaques, and acute myocardial infarctions.⁹⁰ Recently, Hcy was shown to induce MMP-9 levels in mouse macrophages.¹³⁴ Regulated MMP production and activity is important in vascular remodeling and angiogenesis, but HHcy-mediated abnormal activation and production leads to malfunction of macrophages, endothelial cells, and smooth muscle cells, resulting in adverse remodeling and constrictive and stiff arterials with disproportionate collagen deposition.¹³⁵ It remains to be seen to what extent HHcy-mediated MMP-9 induction in macrophages contributes to the progression and rupture of atherosclerotic plaques.

In summary, there is a strong body of evidence that suggests a proatherogenic role for HHcy through modulation of macrophage function at many different levels (Fig. 3): (1) HHcy, by enhancing superoxide ion radicals, activates NF- κ B and enhances MCP-1 production in macrophages, thus enhancing the chances of monocyte binding to arterial endothelium, leading to atherogenesis;^{136–143} (2) HHcy causes monocyte differentiation in the vessel wall;¹²⁴ (3) HHcy enhances expression of CD36, a scavenger receptor for OxLDL, in macrophages, potentially leading to foam cell formation;¹⁴⁴ (4) HHcy stimulates macrophage-derived MMP9 production;¹³¹ and (5) HHcy can promote macrophage polarization to M1¹³⁴ and the switch from M2 to M1 in the presence of lipopolysaccharide.¹⁴⁵

Role of paraoxonases (PON)

High-density lipoprotein (HDL) has an antiatherogenic role. HDL removes excess cholesterol from peripheral tissue and protects against the formation of LDL and thus inhibits OxLDL generation.¹⁴⁶ Although some studies have argued against HDL being atheroprotective,^{147–149} the nature of this lipoprotein and its well-established inverse relationship with atherosclerosis risk strongly indicates otherwise. Some of the anti-inflammatory and antioxidant properties of HDL are attributed to PON1, an HDL-associated protein. PON1 is a calcium-dependent esterase, specifically a paraoxonase, belonging to the PON family of proteins and is strongly associated with HDL.¹⁵⁰ There are three human PON proteins (PON1, PON2, and PON3), all sharing about 65% gene similarity, with PON1 being the predominantly secreted protein found in circulation and PON2 and PON3 limited to intracellular compartments.¹⁵⁰

The PON1 protein confers protection against atherogenesis at multiple levels. Perhaps the most direct and significant antiatherogenic function of PON1 is to protect against oxidative modification (OxLDL) of LDL and subsequent atherosclerotic lesion formation.¹⁵¹ PON1 activity also positively correlates with oxidation of LDL and HDL.¹⁵² Transgenic murine models with high levels of PON1 have fewer lesions when compared to the *ApoE* null mice with normal PON1 levels on a high-fat diet despite having similar cholesterol levels and, in contrast, *Pon1* knockout mice have more atherosclerotic lesions and oxidative stress.^{153, 154} The protective role of PON1 against atherosclerosis is also closely tied to its function within macrophages; although there is no evidence that macrophages express this protein, they express PON2 and PON3.¹⁵⁵ However, macrophages have several binding sites for internalization of PON1.¹⁵⁶ PON1 inhibits monocytes from differentiating into macrophages,¹⁵⁷ most likely due to its strong antioxidant properties and stimulation of

cholesterol efflux when present in HDL, thus preventing activation and differentiation.^{154,158,159} Additionally, PON1 reduces the inflammatory response of macrophages, most likely through binding of SR-BI scavenger receptors for OxLDL, which is independent of cholesterol efflux mechanisms.¹⁶⁰ Moreover, PON1 can reduce cellular oxidative stress as well as the rate of cholesterol biosynthesis after entry into the macrophages.^{161–163} Various mechanisms have been proposed to explain the antioxidant properties of PON1. In macrophages, PON1 reduces total peroxide levels and superoxide production,^{161,163} through inhibition of membrane translocation of the p47phox component, which reduces the NADPH-oxidase activity. Extracellularly, PON1 participates in ternary complexes with HDL and MPO in which it reciprocally inhibits MPO activity and reduces oxidative stress.¹⁶⁴ Recent evidence also suggests that PON1 can directly bind to the atherosclerotic lesion/plaque and can thus suppress foam cell formation.¹⁶⁵ All of these PON1-dependent functions contribute to protection from atherosclerosis.

Given such important anti-atherogenic functions of PON1, it has been proposed as a therapeutic target in prevention or regression of the plaques. In this regard, there are certain factors that modulate PON1 activity and need to be considered before realization of its full therapeutic potential. The diabetic condition,^{166,167} obesity,¹⁶⁸ age,¹⁶⁹ high serum triglycerides,¹⁷⁰ VLDL,¹⁷¹ oxidative stress,¹⁷² and HHcy¹⁷³ all, to certain extent, inhibit PON1-associated paraoxonase, lactonase, and/or esterase activity. Interestingly, all these factors are also risk factors for atherogenesis. Additionally, there are PON1 polymorphisms that produce variability in its activity.¹⁷⁴ In this regard, a combination of statins (cholesterol-lowering drugs that inhibit HMG-CoA reductase), antioxidants, anti-inflammatory agents, Hcy-controlling drugs, and PON1 supplementation should be tailored to the various pathologies (mentioned above) and genetic backgrounds to prevent atherosclerosis. PON1 function is attributed to its paraoxonase, esterase, and lactonase functions in the context of atherogenesis. Studies suggested that PON1's ability to act as a homocysteine thiolactonase may prevent homocysteinylolation of proteins. Reduced PON1 lactonase activity is correlated with enhanced risk for CVD.¹⁷⁵ The lactonase activity also modulates the activity and distribution of several exogenous (drugs such as statins) and endogenous compounds, hence producing modifier effects.¹⁷⁶ The paraoxonase activity is believed to detoxify external toxic organophosphorus compounds.¹⁷⁶ The esterase activity of PON1 is also important in hydrolyzing oxidized lipids (including oxidized phospholipids) present in the lipoproteins as well as atherosclerotic lesions.¹⁵²

PON1 hydrolyzes several metabolites, including homocysteine thiolactone, a toxic by-product whose levels correspond to the plasma Hcy (positive correlation) and folate levels (negative correlation), and thus displays a clear antiatherogenic role.^{177–179} Hcy–thiolactone is generated as a by-product in error-correction reactions during protein biosynthesis when Hcy is selected by methionyl–tRNA synthetase.¹⁸⁰ Like Hcy, Hcy–thiolactone is a risk factor associated with cardiovascular and kidney disease, diabetes, and Alzheimer's disease. Given that HHcy inhibits PON1 activity and generates even more Hcy–thiolactone, there is a much greater potential for inhibition of PON1 activities on macrophage polarization, foam cell formation, and inflammatory profile during HHcy. Hence, it would be valuable to understand the potential ramifications of PON1–HHcy–macrophage interactions. To aid in future studies, we propose a hypothetical perspective on how HHcy might modulate

macrophage function during atherogenesis (Fig. 2). We predict that some of these effects are mediated by HHcy inhibition of PON1 activity.

Role of exercise

Hypertension, diabetes, and hyperlipidemia are the well-known risk factors for atherosclerosis. Regular exercise has the physiological benefits equivalent to those of a multidrug approach with fewer adverse effects in treating these risk factors.¹⁸¹ In addition to these seminal benefits, regular physical activity also confers numerous pleiotropic effects in countering the atherogenic process, such as arterial wall remodeling, plaque size modulation, macrophage function, and regulation of Hcy levels. To realize its full potential (to minimize outcome differences and adverse effects (oxidative stress) and to maximize benefits), certain factors need to be considered. Type of exercise (aerobic or anaerobic), intensity (mild, medium, or heavy) and regularity were reported to cause differential outcomes given the background (physically active or sedentary) and presence of other risk factors. Overall, regular, low/medium-intensity aerobic exercise is beneficial in reducing the incidence of atherosclerosis.

Exercise also has a restorative effect on coronary arteries (the type most likely to experience fatal plaque ruptures), increasing stiffness, decreasing age-related wall-to-lumen ratio, reducing the collagen-to-elastin ratio,¹⁸² and potentially reducing the detrimental effects of HHcy on macrophages.¹¹⁸ In a spontaneous hypertensive rat model, exercising mice had 40% more elastin than sedentary groups despite a lack of significance between control exercise and non-exercise groups, indicating that exercise reverses/prevents elastin depletion.¹⁸³ Also, in this model, exercise normalized elastin, collagen, and SMC content in aortas and significantly enhanced eNOS levels.¹⁸⁴

Exercise also regulates plaque size and its rupturing potential/stability¹⁸⁵ through modulation of matrix content and matrix regulators. In *ApoE* knockout diabetic mice, exercise decreases MMP-2 (degrades collagen, found in SMCs in plaques), MMP-3 (degrades type II collagen, found in SMCs and macrophages in plaque), and MMP-8 (degrades collagens, found in macrophages in plaque) levels as well as IL-6 (expressed by M1 macrophages). Collagen, elastin, and TIMP-2 (inhibitor of MMP-2 and MMP-9) were also increased in parallel with fibrous cap thickness and fewer.¹⁸⁶ In nondiabetic *ApoE* knockout mice, exercise decreased plasma MMP-9 (degrades collagen, found in macrophages in plaques) and increased TIMP-1 (inhibits MMP-2, MMP-9) while increasing collagen and elastin content of plaques and decreasing plaque size.¹⁸⁷ Consistent with this report, recent reports also indicated that exercise reduces MMPs and increases TIMP-1, elastin, and collagen levels in the plaque and thereby confers plaque stability and a reduction in lesion incidence and arterial stenosis.^{188,189} All of these improvements correlate with reduction in macrophages in the plaques,^{188,189} implying that exercise can prevent foam cell formation and reduced macrophage motility, the hallmarks of atherogenesis. Support for such a hypothesis comes from another recent report that indicated an exercise-mediated enhancement in reverse cholesterol transport.¹⁹⁰

Exercise significantly reduced triglyceride levels, but its effects on cholesterol content vary.¹⁸¹ Low-intensity exercise decreases total cholesterol content, but increases OxLDL, HDL and LXR α (most likely through PPAR γ) levels, the latter two being involved with cholesterol efflux and thus having low propensity for atherosclerosis.¹⁹¹ Acute exercise induces an increase in oxidative stress (OxLDL) only in untreated mild hypertensive patients with atherogenic lipid profiles.¹⁹² Recently, aerobic exercise was shown to enhance cholesterol transit from macrophages to the liver (reverse cholesterol transport), potentially conferring prevention and regression of atherosclerosis.¹⁹⁰ Other studies also indicated that exercise can potentially enhance macrophage cholesterol efflux through upregulation of ABCA1 levels.¹⁹³ Together, these results suggest that regular exercise of low intensity but not acute exercise confers benefits in countering atherogenesis. Although exercise and HHcy can affect macrophage function during atherogenesis, their interaction on macrophage function and polarization has not been studied in animal models in the context of atherosclerosis.

Many studies have identified anti-inflammatory function of exercise in countering atherosclerotic risk. Low-intensity exercise was shown to induce M2-type macrophage polarization through PPAR γ activation and modulation of the T_H2 cytokine profile,¹⁹⁴ while there is reduction in M1-specific markers and T_H1-specific cytokine profile, an indication of reduction in the proinflammatory function of macrophages.¹⁹⁴ Exercise also reduces monocyte-derived TNF α (a proinflammatory cytokine) production.¹⁹⁵ Others have also reported that exercise preserves endothelial function and reduces monocyte adhesion and chemotaxis.^{196,197} Exercise also controls blood pressure through regulation of angiotensin II receptor (type I) and thus prevents endothelial dysfunction.¹⁹⁸

Low/moderate-intensity aerobic exercise also counters oxidative stress. Heavy exercise increases PON1 and TBARS (measures of degree of oxidation on lipoproteins) in both physically active and sedentary human subjects, whereas healthy exercise increases PON1 only in physically active subjects but not in sedentary subjects, indicating that regular exercise results in an adaptation of PON1 levels and might contribute to reducing the HHcy-mediated effects in atherogenesis.^{199–201} Exercise was also reported to reduce superoxide production/enhance antioxidant capacity in the arterial wall,^{202,203} potentially reducing the likelihood of OxLDL formation and macrophage accumulation in the arterial intima.

Exercise can reduce HHcy-mediated toxic effects and atherogenesis, either directly by reducing Hcy levels or indirectly by enhancing PON1 levels. Exercise enhanced kidney levels of betaine homocysteine S-methyltransferase, which removes Hcy levels through the nonclassical remethylation pathway by converting Hcy into methionine in a folate-restricted model of HHcy.²⁰⁴ This finding indicated the potential of exercise in countering an HHcy condition originating from genetic mutations in the *MTHFR* gene or dietary insufficiency of folate, where the main classical Hcy remethylation pathway is crippled. Interestingly, HHcy can also limit the exercise capacity;^{70,205,206} hence, it is imperative to correct the HHcy before the exercise regimen to realize its full potential.

In conclusion, regular optimal exercise is highly beneficial in lowering the risk for atherosclerosis through (1) its ability to promote positive arterial wall remodeling, (2) its

ability to enhance serum levels of antioxidants to reduce OxLDL levels and to enhance PON1 activity,¹⁹⁹ (3) its ability to reduce plasma Hcy levels^{118, 204} and triglyceride levels (4) its potential for reducing the foam cell formation through cholesterol efflux from plaques, and (5) its role in suppression of inflammation. Interestingly, all these antiatherogenic subevents are interlinked and can be initiated with changes in the plasma levels of metabolites, such as increases in TG, OxLDL, and Hcy.

Murine models

Although there is considerable human clinical data on atherosclerosis, murine models have been the primary source for learning about the process and about inflammation in general. Mice, rats, and other murine species, however, have vastly different transcriptional and proteomic responses compared to humans when it comes to inflammatory diseases.²⁰⁷ Human and mouse blood differs with respects to leukocyte type: the murine profile is dominated by lymphocytes whereas the human profile is dominated by neutrophils.²⁰⁸ Mice have a significantly greater number of platelets in circulation than humans.²⁰⁹ In fact, there are four times as many platelets in mice, with half of the platelet volume, as in humans.²¹⁰ There are various models for murine atherosclerosis; however, most (if not all) require a combination of *ApoE* and LDL receptor knockouts with a high-fat diet, and even then spontaneous rupture is rare and only occurs with the older mice.^{211,212} When atherosclerosis is achieved, the native macrophages in murine atherosclerotic plaques lack MPO, unlike their human counterparts.²¹³ As such, any atherosclerosis and plaque rupture with murine models is artificial and most likely does not reflect the natural progression of the disease. Altogether, these murine-specific differences demand caution when extrapolating the results from murine disease models to the human disease scenario.

Conclusions and future directions

The accumulated evidence for a role for HHcy in each stage of atherogenesis (endothelial injury, OxLDL formation, monocyte adhesion and chemotaxis into the intima, foam cell formation, and plaque formation) strongly argues for inclusion of HHcy-reducing strategies in the prevention or regression of atherosclerotic plaques. Certain factors, such as genetic background (mutations in Hcy-metabolizing enzymes and PON1 polymorphisms), need to be considered in designing rational HHcy-lowering strategies. Recent evidence has suggested critical roles for macrophages, paraoxonases, and exercise in progression and rupture of atherosclerotic plaques. In light of these findings, it is plausible that HHcy might contribute to aggressive foam cell formation, reduction in macrophage plasticity, enhanced inflammatory milieu, and adverse plaque remodeling, leading to unresolvable self-perpetuating necrotic cores with higher propensity for rupture. Mechanistically, there is a clear need for advancement in deciphering HHcy's role in macrophage polarization and plasticity, OxLDL uptake, scavenger receptor expression, and paraoxonase and lactonase activities within the macrophages. Moreover, whether HHcy alters ER stress in the regulation of macrophage polarity needs to be addressed. In addition to other independent effects on OxLDL formation, macrophage function, cholesterol efflux, paraoxonase level regulation, and arterial wall remodeling during atherogenesis, regular physical activity has

been shown to be beneficial in controlling plasma Hcy levels as well. Hence, exercise could be an obvious choice for countering atherosclerosis.

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Figure 1.

While atherosclerotic plaques can form on any artery in the body, the most prone to rupture form at the bifurcation sites of arteries. Atherosclerosis has been shown to initiate as an inflammatory response from the subendothelial cell layer of the arterial wall due to accumulation of LDL and OxLDL. M-CSF and various other chemoattractants attract circulating monocytes, which bind to V-CAM, P-selectin, and ICAM-1 on the endothelium and infiltrate into the intima. Monocytes differentiate into macrophages and further polarize into either M1 or M2 subtypes. Concurrently, they import OxLDL through surface scavenger receptors and eventually become foam cells. Further lesion progression involves migration of smooth muscle cells (SMCs) into the intima and their proliferation, resulting in the initial visible sign of plaque formation. As the plaque grows, the cells of the plaque, the foam cells and SMCs, die, which leads to extracellular lipid deposits and cell debris at the necrotic core. A continuous cycle of more cell migration, unresolved inflammation, cell proliferation, and cell death contributes to enlargement of the plaque. The macrophages, attempting to ease the plaque burden, produce MMPs that degrade components of the plaque, including the fibrous cap composed of collagen. As the cap thins and the ratio of collagen to elastin increases in disparity, the plaque ruptures, most likely at the shoulder regions, resulting in a thrombus that may or may not be occluding/fatal.

Figure 2.

Schematic diagram showing the HHcy-mediated effects at different stages of atherogenesis. HHcy-induced oxidative stress enhancement is the key player behind all of these influences. The adverse effects of HHcy on OxLDL and PON1 also indirectly contribute to HHcy-induced atherogenesis. Both exercise and H₂S are presumed to antagonize HHcy-induced oxidative stress enhancement, thereby preventing atherogenesis.

Figure 3. Schematic diagram summarizing the HHcy-mediated alterations in macrophage function in the context of atherogenesis.