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Anti-Inflammatory Properties of HDL

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Introduction

High-density lipoproteins (HDLs) are a diverse class of particles with numerous atheroprotective functions, including facilitation of reverse cholesterol transport (RCT), improvement in endothelial function, protection of low-density lipoproteins (LDLs) from oxidation, limitation of hemostasis, and retardation of inflammatory activity related to the vascular wall [1–3]. The antiatherogenic effects of HDL reflect biological properties of HDL subpopulations in addition to absolute plasma levels of HDL-cholesterol (HDL-C) [4]. There are structural differences between different HDL particles, with differentiation of HDL subclasses on the basis of particle size, density, surface charge, lipid and/or protein composition all possible. A key function of HDL is to moderate vascular inflammation, particularly expression of cytokine-induced cellular adhesion molecules, monocyte chemotactic protein-1 (MCP-1), and oxidized phospholipids. Recent evidence supports this anti-inflammatory role as a clinically relevant critical pathway by which HDL reduces atherosclerotic burden.

The Role of Oxidation in Atherogenesis

There is considerable evidence that lipid oxidation within the arterial wall plays a critical role in atherogenesis [2]. Oxidized LDL damages artery wall cells, and HDL limits this LDL-induced cytotoxity by decreasing levels of cholesterol hydroperoxides [5,6]. Brown and Goldstein discovered that acetylated LDL but not native LDL was recognized by "scavenger receptors" instead of LDL receptors resulting in cholesteryl ester accumulation in macrophages and the formation of foam cells [7]. Fogelman and Schecter and colleagues subsequently demonstrated that malondialdehyde, resulting from lipoxygenase-mediated oxidation of arachidonic acid, can alter LDL into a form that is taken up by macrophage scavenger receptors allowing for cholesterol esterification in foam cells [8]. Steinberg and colleagues have demonstrated that endothelial cells were capable of oxidizing LDL *in vitro* into a suitable ligand for macrophage scavenger receptors [9]. LDL is most atherogenic once it has undergone a few modification steps, including oxidation [10]. Several studies have shown that artery walls of animals and humans with atherosclerosis contain oxidatively modified LDL [11–13].

Artery wall cells secrete oxidative waste products into their membranes and into the subendothelial space and "seed" the LDL with reactive oxygen species [14–16]. Navab and colleagues have shown that endothelial and smooth muscle cells in coculture similarly secrete oxidative wastes into their surrounding microenvironments, allowing for the oxidation of LDL [17,18].

Vascular Inflammation Resulting from Lipid Oxidation

Oxidative products of lipid metabolism drive vascular inflammatory pathways involving monocyte recruitment, differentiation into macrophages, and formation of foam cells [19]. Berliner et al. showed that oxidized LDL promotes monocyte chemotactic protein-1 (MCP-1) and monocyte-colony stimulating factor (M-CSF) expression by artery wall cells, and monocyte (but not neutrophil) binding to human aortic endothelial cells [20–24]. For this minimally modified LDL (MM-LDL) to be biologically active requires the oxidation of LDL phospholipids that contain arachidonic acid in the *sn*-2 position [25]. Such oxidized phospholipids are present in atherosclerotic lesions in animals at concentrations expected to be biologically active *in vivo* [25].

The oxidized phospholipids in MM-LDL are recognized by autoantibodies. Witztum and colleagues

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demonstrated that an IgM monoclonal antibody isolated from apoE null mice (EO6) that recognizes the oxidized phospholipids in MM-LDL [26] also binds to epitopes in human and animal atherosclerotic lesions [12,19] and the epitopes of oxidized LDL that are necessary for macrophage binding [26]. The epitope recognized by the EO6 antibody is shared by "natural" T15 anti-phospholipid antibodies that are protective against *Streptoccous pneumoniae* [27]. Furthermore, pneumococcal vaccination protected LDL receptor null mice from atherosclerosis [28]. Thus, antibodies to the oxidized phospholipids found in MM-LDL appear to be "preloaded" in the innate immune system [29].

The Role of HDL as an Antioxidant in Atherogenesis

The enhancement of reverse cholesterol transport (RCT) with HDL, apoA-I and apoA-I mimetics is well-documented [30–33]. However, HDL particles also carry enzymes that retard LDL oxidation, including paraoxonase (PON), platelet-activating factor acetylhydrolase (PAFAH), and lecithin-cholesterol acyltransferase (LCAT) [4,34]. These enzymes degrade proinflammatory, oxidized phospholipids, limiting their accumulation in LDL. In addition, apoA-I can bind oxidized lipids ("seeding molecules") and remove them from LDL [35]. This limits the oxidation of phospholipids within LDL, along with the subsequent inflammatory response of atherosclerosis [35,36]. HDL, apoA-I and apoA-I mimetic peptides have been shown both to limit LDL oxidation in cell-free systems [5,37,38] and the inflammatory response in the artery wall coculture studies of Navab and colleagues [35,36]. This may partly explain why HDL, apoA-I and apoA-I mimetics have been shown to decrease atherosclerotic lesions and improve vascular function in animals [30,39–43] and humans [44–46].

LDL always contains some lipoxygenase pathway products (e.g. HPODE, HPETE) [36,47] In a study of freshly isolated LDL from humans free of vascular disease by Navab et al., the level of these oxidation products did not increase during *in vitro* incubations in the presence of antioxidants, suggesting their presence in LDL *in vivo* [36]. However, when the LDL was incubated in the presence of antioxidants along with apoA-I, the resultant LDL contained less than half as much HPODE and HPETE as at baseline [36]. Prior to these incubations the apoA-I contained no detectable oxidized phospholipids. However, after its incubation with LDL, apoA-I acquired more than half of the HPODE and HPETE that had been in the LDL [36]. After incubation with apoA-I, the LDL was unable to stimulate the production of hydroperoxides from phospholipid or generate a monocyte response in artery wall cocultures [36]. When the lipids within apoA-I that was incubated with the LDL were extracted and added back to the LDL that had been treated with apoA-I, the reconstituted LDL regained these abilities [36].

Consistent with these observations, HDL appears to be a major carrier of lipid hydroperoxides in both humans and mice. HDL from C57BL/6J mice, which are susceptible to atherosclerosis, contains more lipid peroxides than HDL from C3H/HeJ mice, which are resistant to atherosclerosis [48]. Navab and colleagues demonstrated that injection of human apoA-I into C57BL/6J mice inhibited LDL-induced lipid hydroperoxide formation and monocyte chemotaxis [36]. Similarly, following infusion of apoA-I and phospholipid into healthy human volunteers, the ability of their LDL to induce lipid hydroperoxide formation and/or LDL-induced monocyte chemotactic activity was markedly reduced in each of six subjects [36]. Thus, apoA-I has the ability to remove lipid oxidation products from human LDL and significantly lessen the inflammatory potential in both mice and humans [36].

Modifying HDL Anti-Inflammatory Function

HDL's ability to accept lipid hydroperoxides and retard cellular inflammation can change. For example, Van Lenten et al. reported that the acute phase response following elective surgery in humans was associated with a change in HDL from anti-inflammatory to proinflammatory [49]. At the peak of the acute phase response, 3 days after surgery, HDL from the same patient enhanced LDL oxidation and monocyte response in the coculture, i.e. was proinflammatory. However, by one week after surgery the HDL reverted to its usual anti-inflammatory state [49], consistent with an acute phase response. Gabay and Kushner [50] described a "chronic" acute phase response, and Ridker [51] suggested that such a chronic inflammatory state can be seen in humans with persistent elevation in C reactive protein (CRP). Dietary factors may also contribute to chronic vascular inflammation, as demonstrated by Navab et al. in apoE null mice on a chow diet and LDL receptor null mice on a high-fat diet [52].

Changes in HDL anti-inflammatory function may reflect enzymatic changes within HDL. For example, *in vivo*, the absence of the HDL-associated enzyme paraoxonase (PON) resulted in increased LDL oxidation and increased atherosclerosis in a mouse model [53]. Van Lenten et al. demonstrated that the proinflammatory HDL phenotype associated acute phase response in rabbits and humans could be reversed by the addition of either purified PON and PAFAH [49]. The decreased activities of these enzymes in an acute phase response may in turn result in enhanced LDL oxidation and accelerated atherosclerosis.

Gowri and colleagues reported that the ability of the HDL_2 subfraction from healthy humans to retard macrophage-mediated LDL oxidation was positively correlated with HDL_2-associated PAFAH activity [54]. In patients with poorly controlled type 2 diabetes, however, LDL oxidation was significantly ($p < 0.05$) less inhibited than in controls, and did not relate to HDL_2-associated PAFAH activity [54]. Kontush et al. recently showed that a group of patients with hyperalphalipoproteinemia (HALP) had HDL 2a, 2b, 3a, and 3c subfraction concentrations that were up to two-fold higher than normolipidemic controls, but had lower specific antioxidant activity on a unit mass basis, during LDL oxidation [55]. Paraoxonase activity was deficient from all HALP fractions, and levels of PON1, PAFAH, and LCAT all significantly correlated with the antioxidant activity of all HDL fractions [55]. PON1 accounted for approximately 25% of the variation, with PAFAH and LCAT accounting for approximately 12% each [55]. Thus, multiple enzymatic activities within HDL play a role in prevention of LDL oxidation.

One can characterize the inflammatory response of an individual's HDL by its ability to lessen or intensify cellular inflammation and/or oxidation of phospholipids associated with LDL. Navab and colleagues [48] compared both monocyte chemotactic activity (MCA) in a human artery wall coculture and phospholipid oxidation in a cell-free assay (CFA) prior to and after the addition of test HDL. The group designated the "inflammatory index" as the ratio of the MCA (or CFA) before and after the test HDL, with indices less than 1.0 classified as "anti-inflammatory" and those greater than 1.0 classified as "proinflammatory" [48]. Navab reported that the inflammatory/anti-inflammatory properties of HDL from 27 normolipidemic coronary patients clearly separated the patients from 31 age- and gender-matched controls. The patient HDL, in contrast to control HDL, showed pro-inflammatory MCA and CFA results, suggesting that the inflammatory properties of HDL in these patients reflected a "chronic" acute phase response similar to that associated with high-normal CRP levels [51,56].

Ansell et al. [57] studied HDL inflammatory/anti-inflammatory function in two patient groups. Group I consisted of 26 patients who presented with stable coronary heart disease (CHD) or CHD risk equivalents by NCEP ATP-III criteria [58] that were naive to hypolipidemic medication and whose physicians recommended treatment with a statin [57]. The inflammatory/anti-inflammatory properties of HDL from these patients was compared before and six weeks after starting statin therapy. Group II presented with high HDL-cholesterol levels and clinical CHD [57]. The HDL from both groups of patients were compared to that from age- and gender-matched controls [57]. Ansell et al. compared the MCA generated when a standard control LDL was added to a human artery wall cell coculture with and without a test HDL [57]. Ansell et al. also used a cell-free assay in which the oxidized phospholipid PEIPC was added to the fluorescent probe DCFH [57]. PEIPC was used since it is responsible for more than 80% of the LDL-induced MCA in the coculture model.

As shown in the MCA values of Figure 1A, the patients in Group I had pro-inflammatory HDL prior to statin therapy. After six weeks of simvastatin 40 mg/day [57] their HDL was less pro-inflammatory, but was still slightly pro-inflammatory on average. In contrast, the HDL from healthy age- and gender-matched controls was anti-inflammatory. The cell-free assay results were similar (Fig. 1B). The lipid peroxide concentration in the patients' HDL was significantly higher than in the controls' HDL (Fig. 2). Following the six weeks of simvastatin therapy, there was a nonsignificant trend toward lower levels of HDL lipid hydroperoxides ($p = 0.07$) [57]. In Group II, consisting of 20 patients with CHD/risk equivalents whose HDL-cholesterol levels were between 84 mg/dL and 148 mg/dL on no lipid-lowering medication [57], HDL was uniformly pro-inflammatory (Fig. 3).

In Ansell's Group I, only 3 of 26 patients showed low levels of HDL-cholesterol (40 mg/dL) as defined by ATP-III, prior to simvastatin therapy, while 20 of 26 patients had proinflammatory HDL (inflammatory index >1.0) by the coculture assay. All 26 patients had an HDL inflammatory index >0.6 [57]. In contrast, 24 of the 26 controls had a very anti-inflammatory HDL inflammatory index <0.6 [57]. After six weeks of simvastatin therapy, while there was a highly significant reduction in the proinflammatory nature of the HDL from Group I patients, their HDL remained frankly proinflammatory on average (inflammatory index = 1.08) [57].

Group II included patients with CHD despite high HDL-cholesterol levels (95 ± 14 mg/dL). Only one patient had an elevated LDL-cholesterol level (>160 mg/dL), only two patients had elevated triglycerides (>150 mg/dL), and none had diabetes mellitus [57]. Eighteen of these 20 patients had proinflammatory HDL (inflammatory index ≥ 1.0) while only one had an HDL inflammatory index <0.6. Conversely, all 20 of the controls exhibited anti-inflammatory HDL (inflammatory index <0.6) based on MCA [57].

HDL inflammatory index was significantly correlated to HDL-lipid hydroperoxides (LOOH), as determined by both the coculture and the cell-free assays [57]. The assays must measure more than HDL-LOOH, though, since adding control HDL generally produced a low inflammatory index despite the presence of LOOH in this HDL [57]. In fact, only one of the 46 healthy control subjects studied by Ansell and colleagues had an HDL inflammatory

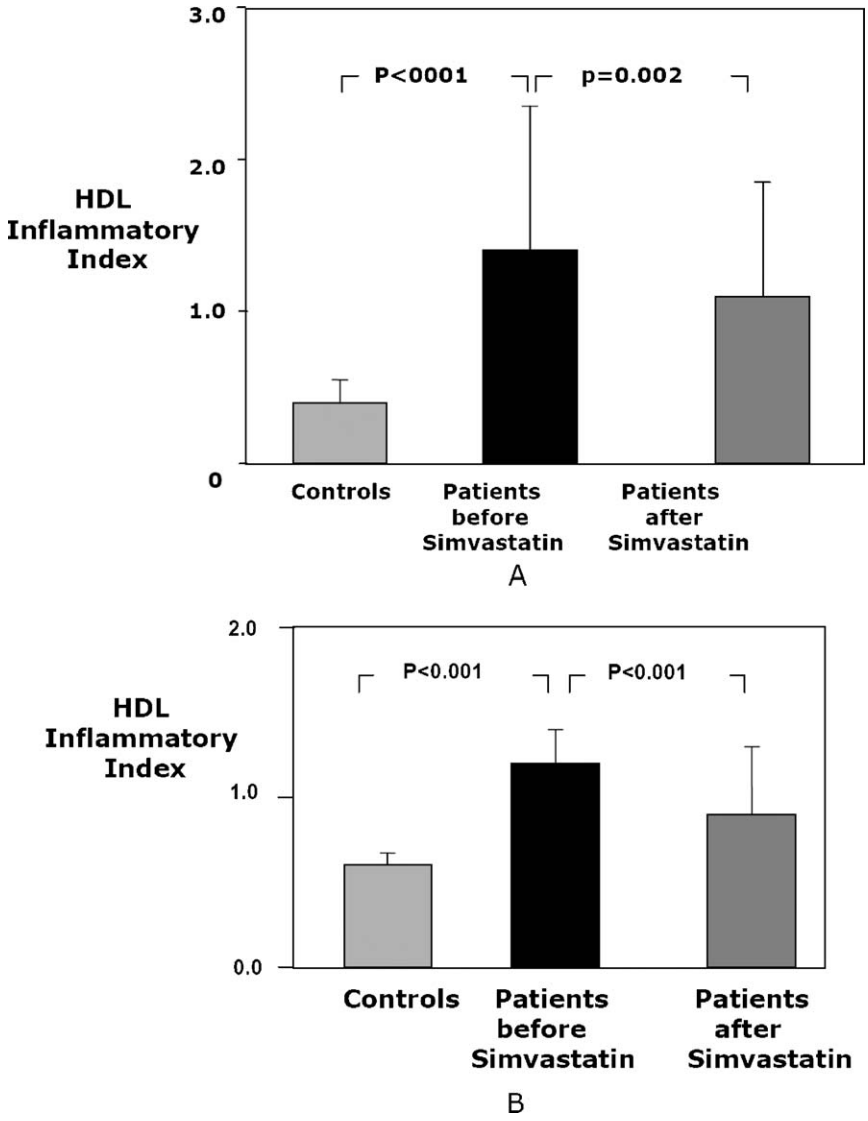

Fig. 1. *HDL inflammatory index assessed by MCA (Panel A) or CFA (Panel B) in 26 patients with CHD or CHD equivalents (Group I), prior to six weeks after simvastatin, compared to healthy age- and gender-matched controls, reproduced with permission from Ansell et al. [48]. See also Ansell et al. [57].*

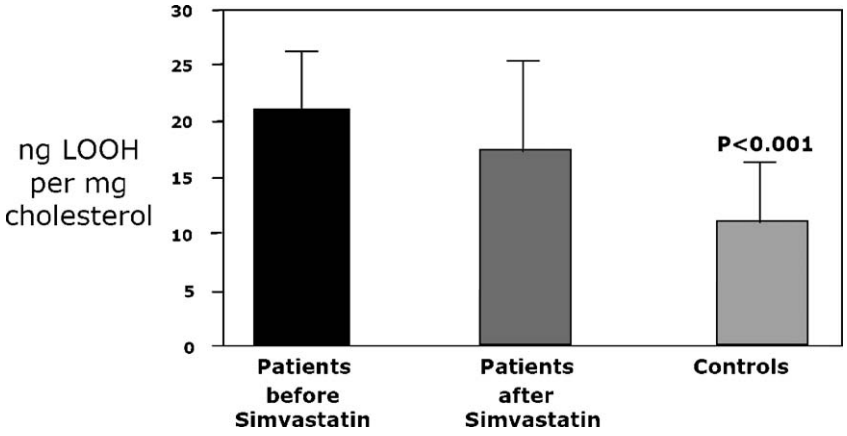

Fig. 2. *HDL lipid hydroperoxide (HDL-LOOH) content for Group I from Ansell et al. [57] patients before and after simvastatin, versus healthy age- and gender-matched controls. Reproduced with permission from Ansell et al. [48].*

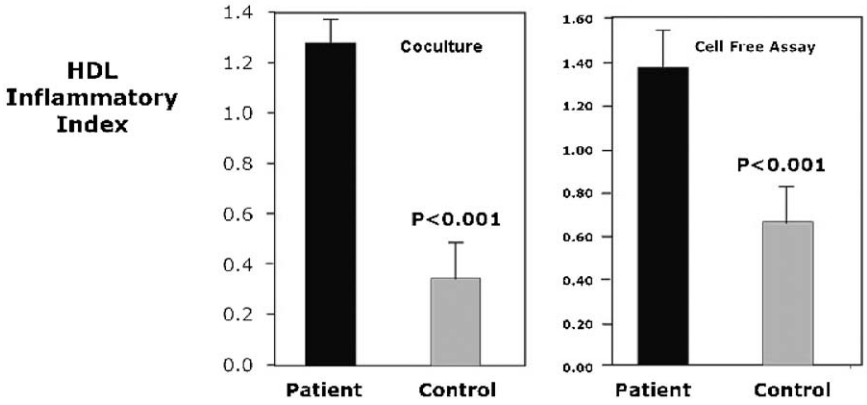

Fig. 3. *HDL inflammatory index in human artery wall cell coculture and in the cell free assay for Ansell's Group II [57]; patients with high levels of HDL-cholesterol and documented CHD versus age and gender matched healthy controls. Values are mean ± S.D. Reproduced with permission from Ansell et al. [48].*

index >1.0 as determined by the cell-free assay. More than just measuring the effects of lipid hydroperoxides, the HDL inflammatory index measures the net contributions of a large number of factors in HDL including oxidized phospholipids, lipid hydroperoxides, PON, PAFAH, LCAT, as well as possibly glutathione peroxidase, apoA-I, apolipoprotein J, serum amyloid A, ceruloplasmin, antioxidant vitamins, and products such as nitrotyrosine, generated by myeloperoxidase [48].

Macdonald et al. [59] reported that it requires more than oxidation of HDL to generate ineffective HDL. In fact, Macdonald and colleagues [59] reported that tyrosyl radical oxidation of mouse HDL promoted heterodimerization of apoAI-AII (tyrHDL), which actually enhanced the ability of mouse HDL to stimulate cholesterol efflux from fibroblasts *in vitro*. When tyrHDL was injected intraperitoneally twice weekly into apoE null mice, 37% less aortic lesion development occurred than in mice treated with control HDL ($P < 0.001$) and 67% less than animals receiving saline ($P < 0.001$) [59]. Bergt, Oram, and Heinecke [60] have suggested that cross-linked heterodimers of apo A-I and apo A-II in tyrosylated HDL appear to be responsible for its ability to promote cholesterol efflux [60,61].

The Relationship Between the Inflammatory and Cholesterol Efflux-Promoting Properties of HDL

HDL's ability to modulate vascular inflammation appears to be linked to its ability to mediate removal of cholesterol from lipid-laden cells. For example, HDL taken from Syrian hamsters experiencing an acute phase reaction due to injection of lipopolysaccharide injection was less able to promote cellular cholesterol efflux than was HDL from control hamsters [62]. In humans with periodontitis associated with PCR evidence of *Actinobacillus Actinomycetemcomitan*, Pussinen et al. [63] reported that HDL mediated cholesterol efflux significantly improved while CRP significantly decreased by 54% with dental treatment. Pussinen et al. [63] concluded that periodontitis likely causes similar changes in HDL metabolism to those during the acute phase response, and that this may diminish the antiatherogenic potency of HDL.

Reddy and colleagues [64] reported that LDL oxidation by human artery wall cells was controlled by the cholesterol content of the cells, which in part was determined by ABCA1 activity. Reddy et al. proposed that the reverse cholesterol transport hypothesis and oxidation hypothesis of atherogenesis might reflect different vantage points of the same process [64].

The data obtained by Navab et al. [48] with D-4F, an oral apoA-I mimetic peptide, are consistent with the proposal by Reddy et al. [64]. A single oral administration of D-4F to cynomologous monkeys reduced plasma and lipoprotein lipid hydroperoxides two hours later. By that time, the monkey HDL contained a significant amount of D-4F within HDL particles. With the ensuing fall in lipoprotein lipid hydroperoxides, there was (1) an improvement in the monkey HDL inflammatory index, (2) a decrease in the ability of the monkey LDL to induce human artery wall cells to produce monocyte chemotactic activity in response to LDL, and (3) a dramatic increase in the ability of the monkey HDL to promote cholesterol efflux from human monocyte macrophages [48].

Summary and Conclusions

The formation of oxidized phospholipids within LDL activates the innate immune system. These oxidized phospholipids are generated from arachidonic acid

via the lipoxygenase and myeloperoxidase pathways. Phospholipid oxidation may also affect reverse cholesterol transport. The HDL inflammatory index and measurements of the levels of lipid oxidation products in lipoproteins including products of the myeloperoxidase pathway may predict susceptibility to atherogenesis. The ability of ApoA-I and apoA-I mimetic peptides to reduce levels of oxidized lipids and also improve reverse cholesterol transport may have therapeutic potential.

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