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Original Contribution

The acute-phase protein serum amyloid A induces endothelial dysfunction that is inhibited by high-density lipoprotein

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ABSTRACT

The acute-phase protein serum amyloid A (SAA) is elevated during inflammation and may be deposited in atheroma where it promotes atherosclerosis. We investigated the proatherogenic effects of SAA on the vascular endothelium and their regulation by high-density lipoprotein (HDL). Exposure of human aortic endothelial cells (HAEC) to SAA (0.25–25 μg/ml) decreased nitric oxide ($^{\bullet}NO$) synthesis/bioavailability, although the endothelial NO synthase monomer-to-dimer ratio was unaffected. SAA (10 μg/ml) stimulated a Ca^{2+} influx linked to apocynin-sensitive superoxide radical anion ($O_2^{\bullet-}$) production. Gene expression for arginase-1, nuclear factor κB (NF-κB), interleukin-8, and tissue factor (TF) increased within 4 h of SAA stimulation. Enzymatically active Arg-1/2 was detected in HAEC cultured with SAA for 24 h. Therefore, in addition to modulating $^{\bullet}NO$ bioavailability by stimulating $O_2^{\bullet-}$ production in the endothelium, SAA modulated vascular L-Arg bioavailability. SAA also diminished relaxation of preconstricted aortic rings induced by acetylcholine, and added superoxide dismutase restored the vascular response. Preincubation of HAEC with HDL (100 or 200, but not 50, μg/ml) before (not after) SAA treatment ameliorated the Ca^{2+} influx and $O_2^{\bullet-}$ production; decreased TF, NF-κB, and Arg-1 gene expression; and preserved overall vascular function. Thus, SAA may promote endothelial dysfunction by modulating $^{\bullet}NO$ and L-Arg bioavailability, and HDL pretreatment may be protective. The relative HDL to SAA concentrations may regulate the proatherogenic properties of SAA on the vascular endothelium.

Functional endothelium is vital for maintenance of vascular homeostasis. It provides a physical barrier to blood components from the subendothelial space [1]. Endothelial dysfunction is a precursor of atherosclerosis [2], essential hypertension [3], and related cardiovascular disease. Endothelium-derived nitric oxide ($^{\bullet}NO$), produced via the action of nitric oxide synthase (eNOS) on L-arginine (L-Arg), regulates vascular tone [4], interactions between platelets [5] or leukocytes [6] with the endothelium, and vascular smooth muscle cell (VSMC) proliferation [7].

Circulating serum amyloid A (SAA) is produced principally by the liver, and levels rise >1000-fold in the acute-phase response; tumor necrosis factor (TNF), interleukin (IL)-1, and IL-6 with endogenous glucocorticoids act as costimulants [8]. SAA induction involves the transcription factors SAA-activating sequence binding factor, NF-κB, NF-IL-6, and Sp1 [9]. Plasma SAA is present as an apolipoprotein of high-density lipoprotein (HDL), largely HDL_3. Elevated levels are evident in chronic diseases, including diabetes, rheumatoid diseases, cancer, and atherosclerosis [10], and predict cardiovascular disease risk [11,12]. Whether SAA is merely an inflammation marker or mediates atherogenesis is debated [11].

SAA is present in atherosclerotic lesions [13], and monocytes/macrophages, VSMC, and EC synthesize SAA, suggesting that its deposition may play a role in atherogenesis. The positive transcoronary gradient of SAA in patients with coronary artery disease, particularly those with acute coronary syndromes [14], confirms localized production. A recent study demonstrated that pathological levels of SAA inhibit endothelium-dependent vasomotor function by increasing EC-derived superoxide radical anion ($O_2^{\bullet-}$) generation, through a process requiring 24 h [15]. Other proinflammatory functions of SAA include induction of IL-6, IL-8, and TNF in neutrophils and promotion of monocyte chemotaxis and adhesion [16,17]. SAA mediates binding of HDL to macrophages and EC [18], impairs HDL's ability to promote cholesterol

Abbreviations: ACh, acetylcholine; L-Arg, L-arginine; Arg-1/2, arginase-1/2; cGMP, guanosine 3′,5′-cyclic monophosphate; DPI, diphenyliodonium; EC, endothelial cell; HAEC, human aortic endothelial cell; HPSS, Hepes-buffered physiological salt solution; MCP-1, monocyte chemotactic protein-1; $^{\bullet}NO$, nitric oxide; ODQ, 1*H*-(1,2,4)-oxadiazolo-(4,3-*a*)-quinoxalin-1-one; $O_2^{\bullet-}$, superoxide anion radical; PBMC, peripheral blood mononuclear cells; PEG–SOD, polyethylene glycol superoxide dismutase conjugate; sGC, soluble guanylyl cyclase; SNP, *S*-nitrosopenicillamine; SAA, serum amyloid A; TF, tissue factor.

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efflux from macrophages [19], and binds vascular proteoglycans [20], a process critical for retention of atherogenic lipoproteins. Paradoxically, SAA incorporation into HDL does not impair HDL's anti-inflammatory action on vascular EC [21], yet SAA can displace apo AI from the lipoprotein [10]. SAA stimulates some early proatherogenic events, including increased proteoglycan synthesis and retention of low-density lipoprotein (LDL)-like particles in the vessel wall of apolipoprotein E-deficient mice that overexpress human SAA [22].

Plasma HDL levels are inversely related to atherosclerotic risk [23]. In addition to its regulation of reverse cholesterol transport, HDL has antioxidant and anti-inflammatory properties that contribute to its activity [24,25]. HDL modulates EC adhesion molecule expression, and via its action on monocyte chemotactic protein-1 (MCP-1), decreases monocyte infiltration into the aortic wall [25]. In support of the idea that HDL limits SAA activity, we have demonstrated that HDL ameliorates tissue factor (TF) [26] and other proinflammatory gene expression in monocytes treated with pathological SAA concentrations [27]. Here we demonstrate that HDL can ameliorate SAA-induced endothelial dysfunction, dependent on the relative concentrations of SAA and HDL. Therefore, circulating levels of SAA and HDL are both potentially important factors that contribute to atherothrombosis.

Experimental procedures

Materials

Most biochemicals were obtained from Sigma (Sydney, Australia) including lipopolysaccharide (LPS; *Escherichia coli* 055:B5). 1*H*-(1,2,4)-Oxadiazole-(4,3-*a*)-quinoxalin-1-one (ODQ), an inhibitor of soluble guanylyl cyclase (sGC), was from Alexis Biochemicals (San Diego, CA, USA). Endothelial cell growth supplements were from Chemicon (Sydney, Australia). Recombinant SAA (PeproTech, Rocky Hill, NJ, USA) is a consensus molecule of the SAA1 and 2 isoforms, used in all previous studies. All reagents and medium were rigorously tested for endotoxin levels using the *Limulus* amebocyte lysate (LAL) buffer and endotoxin standards, visualized with Spectrozyme LAL (American Diagnostica). Reagents were not used if they contained endotoxin levels of >5 pg/ml. We have exhaustively tested recombinant SAA for LPS and find <1 pg LPS/μg SAA/ml. In previous studies we have demonstrated that this low LPS level cannot induce proinflammatory/thrombotic responses in peripheral blood monocytes that are highly sensitive to LPS [14]. Recombinant SAA activity is completely ablated by boiling the protein [26], whereas LPS retains its activity under identical conditions.

Isolation of high-density lipoprotein

HDL was isolated from fresh plasma as described previously [28]. All HDL preparations were desalted using a Sephadex G20 column (Pharmacia, Australia) and the protein content was determined using the bicinchoninic acid assay (Sigma). Preparations were routinely tested for LPS content and any batches containing >5 pg/mg protein were rejected.

Animals

Aortic segments were obtained from male Wistar rats (Animal Resource Centre, Perth, Western Australia) with local ethics committee approval. Animals were anesthetized (isoflurane gas), a thoracotomy was performed to expose the heart, the vasculature was gravity perfused with phosphate-buffered saline (pH 7.4; 150 mM NaCl) fed into the left ventricle, and the aorta was harvested.

Vascular reactivity

Isolated aortae were placed in modified Krebs–Henseleit solution (containing (in mM) 11 D-glucose, 1.2 $MgSO_4$, 12 KH_2PO_4, 4.7 KCl, 120 NaCl, 25 $NaHCO_3$, and 2.5 $CaCl_2 \cdot 2H_2O$) and cut into 5-mm rings [29]. Where required, rings were incubated with 500 U/ml polyethylene glycol superoxide dismutase (PEG–SOD) or 50 mM phosphate-buffered saline (vehicle control) at 4 °C for 12 h before use [30].

Rings were mounted in a Myobath system (World Precision Instruments, Sarasota, FL, USA) containing 15 ml of modified Krebs–Henseleit solution aerated at 37 °C with 5% $CO_{2(g)}$, and individual rings were contracted with phenylephrine (10^{-9}–10^{-5} mol/L). The dose that caused half-maximal contraction in each ring was selected for further studies. Concentration–response curves (10^{-9}–10^{-5} mol/L) to endothelium-dependent (acetylcholine; ACh) or endothelium-independent (*S*-nitrosopenicillamine; SNP) were constructed in the presence of indomethacin (25 μM) with relaxation expressed as a percentage of initial contraction. Mounted rings were pretreated with vehicle (control), SAA (final concentration 0.25, 1, 5, or 1 μg/ml), or 100 μM ODQ for 4 h followed by washing, preconstriction, and relaxation.

To assess HDL effects on vascular responses of SAA-treated vessels, isolated aortic rings were preincubated (30 min; 37 °C) with HDL (final concentrations 0, 10, 50, 100, 200, or 400 μg/ml) before incubation with SAA. After 4 h, vessels exposed sequentially to HDL and then SAA were used to generate cumulative concentration–response curves to ACh. In some studies, HDL was added after 4 h incubation of the aortic segments with SAA.

Cell culture

Human aortic endothelial cells (HAEC; American Type Culture Collection, Manassas, VA, USA) were cultured in F12 Kaighn's nutrient mixture (Invitrogen, Sydney, Australia) containing fetal bovine serum (10% v/v), 100 μg/ml heparin sulfate, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 30 μg/ml endothelial cell growth supplements (Millipore, Sydney, Australia) at 37 °C in 5% $CO_{2(g)}$ as described [31,32].

Confluent HAEC, maximum five passages, were overlaid with serum-free medium containing 100 μM L-Arg and then treated with vehicle (control) or SAA at the indicated concentrations. After 4 h, cells were washed with Hepes-buffered physiological salt solution (HPSS) and exposed to 1 μM ACh. After 15 min, the cells were harvested and centrifuged (38*g*) and the cell pellet and supernatant were separated for analyses outlined below. To assess whether HDL affected SAA stimulation of HAEC, cell preparations were preincubated (30 min, 37 °C) with fresh HDL (0, 10, 50, 100, 200, or 400 μg/ml) before incubation with SAA. In other studies HDL was added to cells after stimulation with SAA. IL-6, IL-8, and MCP-1 were measured using a Bioplex multiprotein array (Bio-Rad, Sydney, Australia). In yet other studies HAEC were cultured as monolayers onto transwells (ThinCerts; 0.4 μm; Interpath Services, Australia) and EC barrier function assessed was by monitoring the time-dependent diffusion of a FITC-labeled dextran substrate [33] (excitation 485; emission 538 nm) in the presence or absence of 10 μg/ml SAA (4 h; 37 °C).

$^{\bullet}$NO accumulation by stimulated HAEC

The accumulation of $^{\bullet}$NO by HAEC was monitored directly with a $^{\bullet}$NO-selective electrode (ISO-NO MkII; World Precision Instruments) coupled to a DUO-18 amplifier and data recorder (DUO-18 version 1.55) as described elsewhere [30]. In some studies, $^{\bullet}$NO was estimated by determining total nitrite in the medium after converting residual nitrate to nitrite as described [34]. Confluent cells were treated with vehicle (control), human serum albumin (1 mg/ml inactive control, containing 446 ± 26 pg LPS/mg protein), or SAA (10 μg/ml, a dose that elicited vascular dysfunction); harvested; and resuspended at $3–4 \times 10^6$ cells/ml in HPSS containing 100 μM L-Arg [30,35]. In some studies, HAEC were preincubated with 50, 100, or 200 μg/ml HDL for 30 min before SAA treatment and harvest. The electrode was preequilibrated in the cell suspension at 20 °C for 30 min before addition of 1 μM ACh, and the peak