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Modification by isolevuglandins, highly reactive γ-ketoaldehydes, deleteriously alters HDL structure and function

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Running Title: *Isolevuglandin causes HDL dysfunction and inflammation*

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ABSTRACT

Cardiovascular disease (CVD) risk depends on HDL function, not HDL-cholesterol (HDL-C). Isolevuglandins (IsoLGs) are lipid dicarbonyls that react with lysine residues of proteins and phosphatidylethanolamine. IsoLG adducts are elevated in atherosclerosis. The consequences of IsoLG modification of HDL have not been studied. We hypothesized that IsoLG modification of apoA-I deleteriously alters HDL function. We determined the effect of IsoLG on HDL structure-function, and whether pentylpyridoxamine (PPM), a dicarbonyl scavenger, can preserve HDL function. IsoLG-adducts in HDL derived from patients with familial hypercholesterolemia (n=10, 233.4±158.3 ng/mg) were found to be significantly higher than in healthy controls (n=7, 90.1±33.4 pg/mg protein). Further, HDL exposed to myeloperoxidase had elevated IsoLG-lysine adducts (5.7 ng/mg protein) compared to unexposed HDL (0.5 ng/mg protein). Preincubation with PPM reduced IsoLG-lysine adducts by 67%, while its inactive analogue pentylpyridoxine (PPO) did not. Addition of IsoLG produced apoA-I and apoA-II crosslinks beginning at 0.3 molar equivalents IsoLG per mol apoA-I (0.3 eq.), while succinylaldehyde and 4-hydroxynonenal (HNE) required 10 and 30 eq. IsoLG increased HDL size, generating a subpopulation of 16-23 nm. 1 eq. IsoLG decreased HDL-mediated ^{3}H-cholesterol efflux from macrophages via ABCA1, which corresponded to a decrease in HDL-apoA-I exchange from 47.4% to only 24.8%. This suggests that IsoLG inhibits apoA-I from disassociating from HDL to interact with ABCA1. Addition of 0.3 eq IsoLG ablated HDL's ability to inhibit LPS-stimulated cytokine expression by macrophages, and increased IL-1β expression by 3.5-fold. The structural-functional effects were partially rescued with PPM scavenging.

Numerous epidemiological studies show that HDL-C is inversely correlated with CVD risk (1-4). However, pharmacological interventions that raise HDL-C have failed to reduce risk (5). Recent evidence suggests that risk for CVD is more closely linked to HDL function than to HDL-C levels (6). Risk factors for CVD including obesity, hypercholesterolemia, hypertension, and chronic kidney disease create an environment of high oxidative stress, generating oxidized lipid species that modify HDL and alter its functional properties (7-9). Since HDL possesses several anti-atherogenic functions, including transport of excess cholesterol from the peripheral cells to the liver for excretion, efflux of

The abbreviations used are: CVD, cardiovascular disease; IsoLG, isolevuglandin ; PE, phosphatidylethanolamine; PPM, pentylpyridoxamine; MPO, myeloperoxidase; FH, familial hypercholesterolemia; Hom, homozygous; Het, heterozygous; PPO; pentylpyridoxine; eq., molar equivalence; HNE, 4-hydroxynonenal; MDA, malondialdehyde;; OPA, o-phthalaldehyde; succ, succinylaldehyde; HAE, HDL-apoA-I exchange

cholesterol from macrophage foam cells, anti-inflammation, and more (10), a loss of any of these functions would likely contribute to disease pathogenesis.

IsoLGs (also known as isoketals) are a family of lipid γ-ketoaldehydes that resemble prostaglandins, and are generated both enzymatically by cyclooxygenases and non-enzymatically by lipid peroxidation in parallel to F_2-isoprostanes during oxidative stress (**Figure 1**). F_2-isoprostanes are enriched in HDL, not LDL (11), and are considered the most reliable biomarker of oxidative damage (12) especially of lipid peroxidation (13). While F_2-isoprostanes are chemically stable, IsoLGs are extremely unstable due to the reactivity of the 1,4-dicarbonyl moiety with primary amines such as the ε-amino groups of lysine residues of proteins as well as headgroups of phosphatidylethanolamines (PEs). The initial reaction of the IsoLG aldehyde forms a Schiff base which undergoes a secondary reaction with the 4-keto group to form irreversible pyrrole adducts. These pyrrole adducts easily oxidize in the presence of oxygen to form stable lactam and hydrolactam adducts. IsoLGs also react with multiple proteins to form pyrrole-pyrrole crosslinks (**Figure 1**). The reaction rate of IsoLG to proteins greatly exceeds that of HNE (14) and malondialdehyde (MDA) (15).

Lipid peroxidation has long been postulated to play a critical role in the pathogenesis of atherosclerosis due to oxidative modification of LDL. Modifications of apoB of LDL by lipid-derived oxidation products lead to unregulated endocytosis of modified LDL resulting in macrophage foam cells. IsoLG-lysine pyrrole adducts are present in oxidized LDL, and in human atherosclerotic lesions (16). IsoLG modified LDL induces macrophage uptake through the same receptor that recognizes oxidized LDL but not acetylated LDL (16). However, removal of lipoproteins containing apoB from plasma only decreases total plasma IsoLG-protein adducts by 20-22% (17), suggesting that most IsoLG adducts in plasma are not associated with LDL or VLDL. Importantly, IsoLG-protein adducts are increased two-fold in patients with atherosclerosis or end-stage renal disease compared to healthy controls (17), and correlate more closely with cardiovascular disease risk than classical risk factors, including LDL and total cholesterol levels (18).

The potential contribution of oxidized HDL to atherogenesis has recently received attention as HDL is not only more oxidizable than LDL (19,20) but also the major acceptor of lipid peroxides in plasma including isoprostanes (19). A consequence is that HDL may be exposed to decomposition products of these oxidized lipids. Once HDL is modified, it not only loses important protective functions but also acquires pro-atherosclerotic properties. Another important pathway of oxidative modification involves reactive intermediates produced by phagocytic white blood cells, the cellular hallmark of inflammation. One potent oxidative enzyme is MPO, which is expressed by activated phagocytes and is found in high levels in human atherosclerotic tissues. MPO uses hydrogen peroxide to generate reactive oxygen and nitrogen species that severely impair HDL function. Because MPO complexes with apoA-I on HDL (21), these impairments are likely due to oxidative targeting of apoA-I (22). We have previously shown that MPO generates IsoLG which can adduct to HDL proteins as well as PEs (23). However, the structural and biological consequences of IsoLG modification of HDL have not been explored.

Because IsoLG is extremely reactive and crosslinks proteins, we hypothesize that modification of HDL proteins (particularly crosslinking of its structural proteins apoA-I and apoA-II) by IsoLG generated under oxidative conditions of atherosclerosis would cause deleterious consequences to HDL particle structure and function. To assess the contribution of IsoLG to HDL dysfunction, our laboratory has developed small molecule scavengers of 1,4-dicarbonyls including 5-*O*-pentyl-pyridoxamine (PPM) which react with IsoLG nearly 2000 times faster than lysines react with IsoLG, thereby inhibiting lysine modification (24). In the current study, we demonstrate that IsoLG mimics the effect of MPO on HDL crosslinking and loss of function and that PPM can prevent MPO-mediated HDL dysfunction. We examine the consequences of IsoLG in crosslinking HDL proteins, particle morphology, and various HDL functions including apoA-I exchange, cholesterol efflux, and protection against inflammation. Further, we test the ability of PPM to preserve HDL and thus protect against dysfunction.

RESULTS

IsoLG-HDL adducts are elevated in familial hypercholesterolemia— While IsoLG-protein adducts were previously found in oxidized LDL, in human atherosclerotic lesions (16), and ~80% of all IsoLG-protein adducts in plasma were not associated with apoB-containing lipoproteins (17), we sought to determine the levels of IsoLG-protein adducts in HDL isolated from patients with hypercholesterolemia and atherosclerosis. We isolated HDL using density-gradient ultracentrifugation from plasma of familial hypercholesterolemic (FH) patients (n=10) and from healthy volunteers (n=7). Two of the FH patients had homozygous (hom) FH and 8 of the subjects had severe heterozygous (het) FH with 6 of the patients (2 homFH and 4 severe hetFH) undergoing regular LDL apheresis. From these patients, plasma was collected prior to LDL apheresis. **Figure 2A** shows total plasma cholesterol levels of controls (189.1±31.6 mg/dl) versus FH (298.6±13.1 mg/dl) prior to HDL isolation. We found that IsoLG-protein adducts were significantly higher ($P<0.05$) in FH (233.4±158.3 pg/mg protein) than in controls (90.1±33.4 pg/mg protein) **(Figure 2B)**. These results demonstrate that IsoLG-adducted HDL are increased in conditions associated with hypercholesterolemia and atherosclerosis.

PPM prevents the generation of IsoLG-protein adducts and apoA-I crosslinking by MPO— We previously showed that e*x vivo* oxidation of HDL by MPO (in the presence of glucose oxidase/glucose/sodium nitrate) generates IsoLG-protein adducts (23). We therefore examined the effects of the 1.4-dicarbonyl scavenger PPM on this MPO-mediated oxidation. We found that PPM, but not its inactive analogue pentylpyridoxine (PPO), significantly reduced IsoLG-protein adducts formed when HDL was exposed to MPO (**Figure 3A**). PPM also dose-dependently inhibited MPO-mediated crosslinking of apoA-I (at 50 and 250 eq. to apoA-I), whereas PPO did not (**Figure 3B**). This data demonstrates that IsoLG contribute to MPO-mediated crosslinking and modification of HDL.

IsoLG crosslinks HDL structural proteins, resulting in HDL of larger size— To further characterize the effects of IsoLG on HDL crosslinking, we exposed HDL to increasing concentration of IsoLG starting from 0.1 molar equivalents of IsoLG per mol apoA-I (0.1 eq.) to 3 eq. IsoLG. This range of IsoLG concentrations yielded approximately the expected level of IsoLG-lysine adducts seen *in vivo*. Modification of HDL by 0.3 eq. IsoLG resulted in 242±120 pg/mg IsoLG-lysine, which approximates adducts seen in HDL derived from human FH patients (**Figure 2B**). Modification of HDL by 3 eq. IsoLG resulted in 1936±509 pg/mg IsoLG-lysines, which is below the level produced by ex vivo modification by MPO (**Figure 3A**). We also confirmed that even with the highest concentration of IsoLG used (3 eq.) that no unreacted IsoLG was present in the flow-through when the HDL preparation was filtered through a 10 kD molecular weight cutoff filter (**Supplementary Figure 1A**).

We found that IsoLG dose-dependently crosslinked proteins in HDL starting at ~0.3 eq. (**Figure 4A**). 0.3 eq. IsoLG produced apoA-I immunoreactive bands with molecular weight higher than that of apoA-I monomer and consistent with possible apoA-I dimers and trimers. Higher IsoLG concentrations produced high molecular weight bands that would be consistent with apoA-I crosslinking to additional proteins. PPM blocked crosslinking, but the inactive analog PPO did not (**Figure 4B**). The higher molecular weight bands are seen in Coomassie Blue stained protein gels of IsoLG-modified HDL as well as IsoLG-modified synthetic apoA-I particles containing only recombinant apoA-I as the protein **(Supplementary Figure 2A)**. That the apoA-I antibody detects higher molecular weight bands in the synthetic apoA-I particles strongly supports the notion that the higher weight bands in native HDL modified by IsoLG represent apoA-I oligomers as well as apoA-I crosslinks to other proteins **(Supplementary Figure 2B)**.

Examination of HDL by transmission electron microscopy to quantify particle size showed that IsoLG modification produced larger HDL particles (**Figure 4C-D)**. Unmodified control HDL consisted of small round HDL with mean diameters of 9.50 ± 2.91 nm. At 3 eq. IsoLG, HDL particles consisted of two size distributions: 5-13 nm and 15-23 nm, which was significantly different from unmodified HDL ($P<0.0001$). These results show that IsoLG modification increases HDL particle size as well as crosslinking its structural proteins.

IsoLG is more reactive than other lipid aldehydes in adducting to lysine residues and crosslinking HDL apolipoproteins— To assess to what

extent the structural features of IsoLG uniquely contributed to its effect on HDL, we compared it to two other related lipid aldehydes: 4-hydoxynonenal (HNE, a widely studied α,β-unsaturated aldehyde) and succinaldehyde (a 1,4-dicarbonyl lacking the alkyl and carboxylate tails present in IsoLG) (**Figure 5A)**. We compared the ability of these three lipid aldehydes to modify lysine residues on HDL using o-phthalaldehyde (OPA) to detect available lysines. OPA also detects the headgroups of PEs, but these are in much lower abundance than lysyl residues. Significantly lower molar equivalents of IsoLG than HNE or succinaldehyde were required to modify HDL (**Figure 5B**). For example, 10 eq, IsoLG modified ~50% of the available lysines of HDL, while 10 eq. succinaldehyde only modified ~20% of available lysines, and 10 eq. HNE failed to modify HDL.

Furthermore, IsoLG crosslinks HDL at 10-30 times lower concentrations than HNE and succinylaldehyde (**Figure 5C**). Similar to its effects in purified HDL, addition of IsoLG to plasma resulted in crosslinked apoA-I at a much lower concentration (10 μM) relative to other lipid aldehydes (0.6 mM for acrolein; 5 mM for HNE) (25) (**Figure 5D**). These data demonstrate that IsoLG is far more potent than α,β-unsaturated aldehdydes or non-substituted 1,4-dicarbonyls at modifying lysines and crosslinking HDL proteins.

IsoLG-modified HDL have lower HDL - apoA-I exchange (HAE) and cholesterol efflux to macrophages—ApoA-I exchanges freely with HDL, but not with VLDL, LDL, or albumin. Reduced exchangeability of apoA-I on HDL is associated with atherosclerosis in animal models and in acute coronary syndrome patients (26). Oxidation of apoA-I by MPO also reduces the rate of HAE concomitant with diminished cholesterol efflux capacity (26). Because MPO produced IsoLG (**Figure 3A**) which heavily crosslinked HDL proteins such as apoA-I (**Figure 4A**), we examined if IsoLG modification altered the exchangeability of apoA-I from HDL using the method of Borja, et al (26). While unmodified HDL had an HAE rate of 47.4±1.6%, IsoLG dose-dependently decreased HAE, so that HDL exposed to 1 eq. IsoLG had an HAE rate of 28.9±7.6% ($P<0.01$) (**Figure 6A**).

With the dramatic decrease in HAE in IsoLG-modified HDL, we speculated that HDL with IsoLG modified apoA-I would be less efficient in cholesterol mobilization from macrophages. Thus, we examined cholesterol efflux using apoE$^{-/-}$ macrophages (27). Use of apoE$^{-/-}$ macrophages, rather than wild-type macrophages, provides strict assessment of the effect of IsoLG modification on HDL dependent efflux, as the apoE endogenously produced by wild-type macrophages promotes some cholesterol efflux even in the absence of acceptors such as HDL or apoA-I (28-30). IsoLG modification dose-dependently decreased the ability of HDL to efflux cholesterol (**Figure 6B**), with the same concentration of IsoLG that crosslinked HDL apolipoproteins and decreased HAE. 30 μM PPM (100-fold excess) prevented the decrease in cholesterol efflux induced by 3 eq. IsoLG (**Figure 6B**).

IsoLG modification and crosslinking of HDL can potentially affect proteins involved in promoting cholesterol efflux such as lecithin:cholesterol-acyltranferase (LCAT) (31). Immunoblotting of IsoLG modified HDL did not reveal significant changes in the molecular weight of LCAT immunoreactive bands, suggesting IsoLG does not crosslink LCAT (**Supplementary Figure 3A**). However, it may be that anti-LCAT antibody does not recognize crosslinked proteins. We therefore measured LCAT activity, and found that IsoLG modification of HDL dose-dependently decreases phospholipase A2 activity, but not LCAT activity **(Supplementary Figure 3B)**. Thus IsoLG modification of LCAT does not appear to significantly contribute to the changes in cholesterol efflux seen in our experiments.

To assess whether IsoLG modification primarily disrupted ABCA1 mediated cholesterol efflux to HDL, we employed probucol, an inhibitor of ABCA1-mediated cholesterol efflux that does not interfere with ABCG1 or SR-BI mediated efflux (32). The efflux capacity of IsoLG-modified HDL was only 78.2±9.4% that of unmodified HDL (**Figure 6C**). Control HDL efflux capacity was reduced to 55.4±10.9% original capacity in presence of probucol, and IsoLG-modification of HDL did not further reduce efflux capacity (56.7±11.4%) (**Figure 6C**). This suggests that IsoLG modification of HDL predominantly inhibits the ABCA1 mediated pathway. This is further supported by experiments examining cholesterol efflux to lipid-poor apoA-I, which promotes macrophage cholesterol efflux mainly through ABCA1. IsoLG-modified apoA-I

had significantly reduced cholesterol efflux capacity (31.3±10.3%) compared to unmodified apoA-I (**Figure 6D**). Probucol reduced efflux in unmodified apoA-I to 27.4±9.8%. and IsoLG-modification did not further reduce efflux (27.3±10.9%) (**Figure 6D**). Taken together, the results indicate that IsoLG modification predominantly impairs ABCA1-mediated cholesterol efflux rather than that of other pathways such as ABCG1 or SRBI.

IsoLG-modified HDL induces a synergistic pro-inflammatory phenotype with LPS in macrophages—HDL serves various anti-inflammatory functions including inhibiting the toll-like receptor induced pro-inflammatory cytokine response in macrophages at a transcriptional level (33). We therefore tested whether IsoLG would inhibit HDL's ability to prevent LPS-induced inflammatory response in apoE$^{-/-}$ macrophages. ApoE$^{-/-}$ macrophages were used in these studies to allow direct comparison to efflux studies of the concentrations of IsoLG that altered effects. Coincubation of unmodified HDL with LPS resulted in a 85.5±10.3%, 54.9±21.3%, and 43.6±20.1% inhibition of the LPS-induced expression of *Tnf*, *IL-1β*, and *IL-6*, respectively (**Figure 7A**). Preincubation of macrophages with unmodified HDL, followed by subsequent removal of this HDL and then exposure to LPS did not result in inhibition of LPS induced *Tnf*, *IL-1β*, and *IL-6* expression, consistent with previous observations (34) and the concept that concurrent interaction with macrophages is needed for HDL to inhibit these effects of LPS (**Supplemental Figure 4A**). Coincubation of LPS with HDL modified with 0.3 eq. IsoLG ablated its ability to prevent *Tnf* expression, and even induced a greater expression of *IL-1β* and *IL-6* than LPS-activation alone (365.8±184% and 255.8±90.9% respectively) (**Figure 7A**). This pro-inflammatory phenotype was not seen with treatments with HNE nor succinaldehyde modified HDL (**Figure 7B**). These results show that IsoLG-modified HDL induces a pro-inflammatory phenotype at a concentration of IsoLG that is much lower than that needed to invoke crosslinking of HDL apolipoproteins. Inclusion of PPM completely eliminated the loss of HDL's inhibitory effect on LPS that is seen when HDL is incubated with IsoLG (**Figure 7C**).

The augmentation in cytokine release seen during co-incubation of LPS and IsoLG-HDL raised the possibility that IsoLG modification not only disrupted the ability of HDL to inhibit LPS signaling, but that IsoLG-HDL independently stimulated cytokine signaling. Previous studies found that MPO-oxidized HDL activates pro-inflammatory signaling in endothelial cells, with its effects largely attributed to modified apoA-I protein (35) and that IsoLG-modified PE induce proinflammatory signaling in macrophages in the absence of LPS (36). However, in the absence of LPS, IsoLG-modified HDL does not induce *Tnf*, *Il-1β*, or *Il-6* expression (**Supplementary Figure 4B)**. Furthermore, priming macrophages with IsoLG-modified HDL followed by removal of the HDL and subsequent LPS stimulation did not augment cytokine response (**Supplemental Figure 4A**). Therefore, IsoLG modified PEs in HDL is unlikely responsible for the proinflammatory effect of IsoLG-modified HDL, since IsoLG-modified PEs can induce inflammatory signaling even in the absence of LPS. Taken together, the data demonstrate that IsoLG-modified HDL is not only dysfunctional in preventing LPS-induced macrophage activation, but also synergizes with LPS to induce a more significant inflammatory response..

DISCUSSION

Growing evidence supports the notion that modifications of HDL proteins play a major role in the pathogenesis of atherosclerosis (8,37). Elevated IsoLG protein adducts had been shown in atherosclerosis, but their formation on HDL and resulting consequences have not been studied. In the present study, we demonstrate for the first time that IsoLG protein adducts are elevated in HDL derived from patients with hypercholesterolemia compared to healthy controls, indicating that significant IsoLG-adduct formation on HDL occurs in conditions that promote atherosclerosis. We also show that the crosslinking of HDL that is induced when MPO associates with HDL, can be blocked by a dicarbonyl scavenger. Low concentrations of IsoLG can crosslink apoA-I, the major structural protein of HDL, and generates a HDL subpopulation of larger size, impairs HDL remodeling, cholesterol efflux, and its anti-inflammatory function, and further augments the inflammatory response of activated macrophages. In addition, we demonstrate the potential of dicarbonyl scavengers such as PPM as an anti-atherosclerotic strategy to preserve the HDL particle and prevent HDL dysfunction.

MPO participates in direct protein oxidation, nitration, or chlorination as well as initiates lipid peroxidation in vivo (38-40). Elevated levels of MPO are present in patients with angiographic evidence of CVD (41) and predict risk for myocardial infarction, revascularization, and cardiac death in subjects presenting with chest pain or acute coronary syndrome (42,43). MPO binds to apoA-I and thus directly targets HDL within the human atheroma (44). We verified that IsoLG-protein adducts form in HDL with MPO oxidation, which likely contributes to MPO-mediated crosslinking of HDL proteins. The fact that PPM can prevent IsoLG-protein adduct formation and protein crosslinking as detected by our mass spectrometry analyses and by our immunoblots illustrates the contribution of IsoLG in MPO-mediated oxidation events within the atherosclerotic lesion.

In human plasma, HDL is a heterogeneous collection of particles ranging from 7-12 nm in diameter and 1.063-1.21 g/ml in density. Mass spectrometry identifications report up to 204 different proteins that associate with HDL (45). Approximately 70% of total HDL protein is apoA-I, a 28 kDa apolipoprotein associated with essentially every HDL particle. The second most abundant protein is apoA-II, which comprises 15-20% total HDL protein but is not present in all HDL particles. ApoA-I and apoA-II are the scaffold proteins of HDL that primarily determine particle structure. We demonstrate that IsoLG at very low concentrations (0.3 molar equivalent to apoA-I) crosslinks apoA-I to produce dimers and trimers, which can be prevented by PPM. Modification of HDL by above 1 eq. IsoLG produces multimers of apoA-I/apoA-II and likely crosslinks with other proteins in the HDL proteome.

Concomitant to protein crosslinking, IsoLG modification increased the size of HDL, from an average size range of what is designated to be “medium” to “large” HDL (8.3-10.2nm) to the appearance of “very large” HDL particles (above 10.3-13.5nm). An increase in discoidal HDL particle diameter beyond 10 nm is associated with incorporation of more apoA-I molecules (46). The presence of apoA-II in discoidal apoA-I/A-II containing HDL has been reported to alter the conformation of apoA-I in a site specific manner (47), which could potentially hinder the remodeling of the HDL particles (48). It is likely that the extensive crosslinking of apoA-I and apoA-II by IsoLG caused apolipoprotein aggregates and therefore particle fusion. Crosslinking of apoA-II to apoA-I may also hinder HDL remodeling. These perturbations to HDL remodeling and the formation of very large particles has detrimental consequences, such as the inability to mobilize intracellular cholesterol depots (49) or interact with macrophage ABCA1 (50) to promote cholesterol efflux.

When we measured the rate of HDL-apoA-I exchange, we found that IsoLG dose-dependently reduced the conformational adaptability of apoA-I and thus inhibited HDL remodeling. Previously, decreases in HAE were observed in atherosclerotic animal models as well as human subjects with acute coronary syndrome and metabolic syndrome (26), Type I diabetes (51), metabolic syndrome (52), sickle cell anemia (53), and HIV (54). The decrease in HAE appears to be linked to oxidative damage to HDL and sometimes correlated with loss of other HDL functions, such as cholesterol efflux, since the ability of apoA-I to exchange between lipid-associated and lipid-free states is critical for efficient cholesterol efflux via ABCA1 (55).

We found that IsoLG modification of HDL dose dependently decreased cholesterol efflux from cholesterol-loaded apoE$^{-/-}$ macrophages. The use of apoE$^{-/-}$ macrophages in our studies allowed us to strictly measure HDL-dependent efflux, since apoE endogenously produced by wild-type macrophages promotes ABCA1 dependent cholesterol efflux even in the absence of HDL(28-30). The concentration of IsoLG needed to cause a significant decrease in efflux correlated with apolipoprotein crosslinking as well as the decrease in HDL-apoA-I exchange. These observations support the notion that crosslinking of HDL scaffold proteins alters their conformational adaptability, likely impairing the ability of lipid-free/poor apoA-I to exchange off HDL particles, which is required to elicit efflux of cholesterol via ABCA1 (56,57). Indeed, that probucol (which blocks ABCA1- but not ABCG1-mediated efflux) did not further inhibit efflux in the presence of IsoLG modification supports the notion that the main pathway of cholesterol efflux affected by IsoLG modification of HDL is via ABCA1. The association between HDL protein crosslinking by oxidative modifications and defects in cholesterol efflux have been previously reported in HDL exposed to

copper (58,59), modified by MDA (58), or exposed to MPO (44,60,61). However, not all endogenous crosslinkers of HDL proteins impair function, as HDL apolipoproteins crosslinked by exposure to peroxidase-generated tyrosyl radicals appear to enhance the ability of HDL to facilitate cholesterol efflux (62) which is mediated by apoA-I/apoA-II heterodimers (63).

In addition to cholesterol efflux functions, HDL also protects against infection and inflammation (64). One of the key defense functions of HDL is its ability to neutralize toxic effects of LPS and other bacterial products, which in turn inhibit inflammatory responses in atherosclerosis (65-67). Presumably, modifications of apoA-I or HDL would result in a decrease in function, such as loss of binding ability to LPS (68). We found that modification of HDL at concentrations that were insufficient to induce crosslinking were nevertheless sufficient to render it unable to protect against LPS stimulated inflammatory cytokine response in apoE$^{-/-}$ macrophages. Interestingly, cytokine expression of *Il-1β* and *Il-6* were dramatically higher than LPS induction alone, suggesting that IsoLG-modification of HDL did not simply disrupt the neutralization ability of HDL but synergized with LPS to produce a greater pro-inflammatory phenotype. IsoLG has been previously shown to exert potent inflammatory effects, particularly in activating endothelial cells (69), macrophages (36), and dendritic cells (70). IsoLG-modified PE has recently been shown to induce proinflammatory signaling in macrophages in the absence of LPS (36). The observation that IsoLG-modified HDL did not induce an increase in pro-inflammatory cytokine expression in the absence of LPS suggested that IsoLG-modified PE in HDL was not responsible for the effect. However, IsoLG modification of various HDL components using reconstituted HDL systems will be studied in the future.

The low level of modification by IsoLG needed to promote HDL protein crosslinking, structural and morphological changes, as well as changes in HDL function (especially compared to other known reactive lipid aldehydes) demonstrates that minor lipid peroxidation events in atherosclerosis are sufficient to significantly reduce the levels of functional HDL particles. The ability of scavengers such as PPM to block the ability of 1,4-dicarbonyls including IsoLG to modify proteins and to preserve the HDL particle demonstrates the therapeutic potential of these scavengers in the treatment of atherosclerosis.

EXPERIMENTAL PROCEDURES

Materials—Chemicals required for the synthesis of 4-hydroxyl-2(E)-nonenal (HNE) and succinylaldehyde were purchased from Aldrich (Milwaukee, WI). Reagents for SDS-PAGE and immunoblotting were from Novex by Life Technologies (Carlsbad, CA). Materials used for cell culture were from Gibco by Life Technologies (Grand Island, NY). OPA reagent was purchased from Thermo Scientific (Rockford, IL). [1,2-^{3}H(N)]-cholesterol was purchased from Perkin-Elmer Life Sciences. ApoA-I mouse/human (5F4) monoclonal antibody was purchased from Cell Signaling Technology (Danvers, MA). ApoA-II human (EPR2913) monoclonal antibody was purchased from Abcam (Cambridge, MA). RNEasy Mini kit was purchased from Qiagen (Hilden, Germany). iQ SYBR Green Supermix and iScript cDNA Synthesis kit were purchased from Bio-Rad Laboratories (Hercules, CA).

Plasma from FH patients and healthy controls – Ethylenediaminetetraacetic acid plasma was isolated from the blood of FH patients (n=10), 8 of which had heterozygous FH and 2 had homozygous FH. The 2 homozygous FH and 4 of the heterozygous FH underwent regular LDL apheresis and blood was collected prior to LDL apheresis. Control plasma was isolated from blood of healthy volunteers (n=7). The study was approved by the Vanderbilt University Institutional Review Board (IRB), and all participants gave their written informed consent.

Animals – Breeding pairs of homozygous ApoE$^{-/-}$ mice on a C57BL/6J background (strain 002052) were purchased from Jackson Laboratories (Bar Harbor, ME) at 12 weeks old, and housed in the Vanderbilt University animal facility in a 12-hour light/12-hour dark cycle. The animals were maintained on standard rodent chow (LabDiet 5001) with free access to water. Progeny of the breeding pairs were at least 8 weeks of age before harvest of macrophages (described below). All pro-

cedures were approved by the Vanderbilt University Institutional Animal Care and Use Committee (IACUC).

Chemical Synthesis of IsoLG, 4-HNE, and Succinylaldehyde—15-E_2-IsoLG was synthesized as previously described by organic synthesis (71). 15-E_2-IsoLG is one of eight regioisomers potentially generated by peroxidation of arachidonic acid. The 15- and 5- series of IsoLGs are expected to form in greater abundance than the 8- or 12- series. 15-E_2-IsoLG is also chemically identical to levuglandin E_2 (LGE2) formed nonenzymatically from prostaglandin H_2. For these reasons, 15-E_2-IsoLG is the most widely used regioisomer of IsoLG for studies. 4-HNE was synthesized using the Gardner's procedure (72). Both carbonyls were dissolved in DMSO and prepared as a 10 mM stock and stored as small aliquots in -80ºC until use. Fresh succinylaldehyde was synthesized before each experiment from dimethyl tetrahydrofuran as previously described (73). Fresh working solutions were prepared before each assay and diluted in water to appropriate concentrations.

MPO oxidation of purified human HDL and measurement of IsoLG— HDL obtained from fasting healthy subjects was isolated by density gradient ultracentrifugation and dialyzed into PBS to eliminate residual Tris buffer or other primary amines that would react with the lipid aldehydes and/or their protein adducts. HDL was oxidized by MPO as previously described (23,74). Briefly, HDL was incubated at 37 °C in 50 mM sodium phosphate (pH 7.4), 200 μM diethylenetriaminepentaacetic acid, 57 nM MPO, 100 μg/ml glucose, 20 ng/ml glucose oxidase, and 0.05 mM $NaNO_2$ overnight. Scavengers PPM and its inactive precursor PPO were synthesized as described (75) and solubilized in water. HDL was incubated for 30 minutes at 37ºC before addition of MPO. Quantitation of lysine modification of HDL by IsoLG was performed by first subjecting an aliquot of the preparation to proteolysis with Pronase and aminopeptidase M, and then measuring the amount of IsoLG-lysyl-lactam (the most prominent species of IsoLG modification generated under these conditions) by stable isotope dilution LC/MS/MS as previously described (76).

Lipid aldehyde modification of HDL and the use of scavengers—HDL was exposed to various concentrations of lipid aldehydes at 37ºC overnight to guarantee a complete reaction to form a stable end product. Control HDL was treated similarly in the absence of aldehydes. HDL preparations were diluted with DMEM for incubation with the macrophages. For experiments involving the use of scavengers, PPM and PPO solubilized in water was incubated with HDL for 30 minutes at 37ºC before addition of IsoLG.

Characterization of apolipoprotein crosslinking of modified HDL—HDL apolipoprotein crosslinking was assessed by SDS-PAGE performed under reducing conditions with Invitrogen's gel electrophoresis and transfer system. 4-20% Tris gradient gels were used. Western blot analyses were carried out using polyclonal antibodies specific for human apoA-I and apoA-II.

Characterization of lysine adduction— OPA is a primary amine-reactive fluorescent detection reagent that is used to detect free lysines in HDL (77,78). The procedure was performed according to the manufacturer's instructions (Thermo Scientific) using HDL modified by lipid aldehydes as described above and adapted to 96-well plates. % lysine adduction was calculated as fluorescence of modified HDL / unmodified HDL x 100.

Measurement of HDL morphology and size— Negative stain preparations were prepared from suspensions of particles. The particles were adhered to formvar/carbon-coated grids by floating the grids on top of a drop of the suspension for 45 seconds – 1 minute. The grid was removed from the drop and excess fluid wicked away with filter paper. The particles were then negatively stained by floating the grid with particles on a drop of 1% phosphotungstic acid at pH 5.0 for 45 seconds. Excess stain was removed by wicking with filter paper. The negatively stained particles were imaged by electron microscopy using an FEI T-12 (ThermoFisher) EM operated at 100 keV. For quantitation, 100 particles for each condition were arbitrarily chosen using an unbiased sampling scheme. The particles were chosen from at least 3 separate preparations for each condition. The diameters were measured from the two-dimensional images using

an unbiased algorithm that arbitrarily selected a different angle for each measurement.

HDL-ApoA-I exchange—HDL samples prepared by adding 15 µL 3 mg/mL spin-labeled apoA-I probe to 45 µL 1 mg/mL HDL and drawn into an EPR-compatible capillary tube (VWR) (26). EPR measurements were performed using a Bruker eScan EPR spectrometer outfitted with temperature controller (Noxygen). Samples were incubated for 15 minutes at 37 ˚C and then scanned at 37 ˚C. The peak amplitude of the nitroxide signal from the apoA-I probe in the sample (3462-3470 Gauss) was compared to the peak amplitude of a proprietary internal standard (3507-3515 Gauss) provided by Bruker. The internal standard is contained within the eScan spectrometer cavity and does not contact the sample. Since the y-axis of the EPR spectrometer is measured in arbitrary units, measuring the sample against a fixed internal standard facilitates normalization of the response. HAE activity represents the sample : internal standard signal ratio at 37 ˚C. The maximal % HAE activity was calculated by comparing HAE activity to a standard curve ranging in the degree of probe lipid-associated signal. Experiments were repeated two separate times. All samples were read in triplicate and averaged.

Cell culture— Male and female apoE$^{-/-}$ mice (C57/BL genetic background) were injected intraperitoneally with 3% thioglycolate and the macrophages were harvested by peritoneal lavage after four days. Cells were maintained in 24-well plates in DMEM with 10% (v/v) fetal bovine serum and penicillin-streptomycin at 100 units/mL and 100 µg/mL respectively.

Cholesterol efflux—Efflux was assessed by the isotopic method (79). Loading medium was prepared to consist of DMEM containing 100 µg/ml acetylated LDL with 6 µCi ^{3}H-cholesterol/ml. After equilibration for 30 minutes at 37ºC, loading medium was added to cells for 48 h. After 48h, the cells were incubated for 1h with DMEM containing 0.1% bovine serum albumin so that surface-bound acetylated LDL was internalized and processed. Cells were washed and incubated with efflux medium, which contained DMEM with 35 µg/mL HDL samples. Experiments involving probucol followed the same procedure except that 10 µM probucol was added to the cells 1h before treatment with HDL samples. After 4h incubation, supernatants were collected, vacuum filtered, and prepared for β-scintillation counting.

Macrophage inflammation—Cells derived from female mice were incubated overnight in DMEM containing 0.5% FBS and 1% penicillin-streptomycin. The cells were washed two times with HBSS and then incubated for 4h with DMEM alone or containing 100 ng/ml LPS with or without the HDL preparations (50µg/ml). The cells were lysed, mRNA harvested, and the cDNA synthesized. qPCR was performed with the following primer pairs: *Tnf* forward (5'-CCATTCCTGAGTTCTGCAAAG -3'); *Tnf* reverse (5'-GCAAATATAAATAGAGGGGGGC-3'); *Il-1β* forward (5'-TCCAGGATGAGGACATGAGCA-3'); *Il-1β* reverse (5'-GAACGTCACACACCAGCA-3'); *Il-6* forward (5'-TAGTCCTTCCTACCCCAATTTCC-3'); *Il-6* reverse (5'-TTGGTCCTTAGCCACTCCTTCC-3').

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Conflict of interest – Drs. Davies and Linton are inventors on a patent application for the use of PPM and related dicarbonyl scavengers for the treatment of cardiovascular disease. MNO is a founder of and owns a significant stake in Seer Biologics, Inc., which could stand to benefit from the research described here. This in no way influenced the thoroughness, stringency, interpretation and presentation of his contribution to this manuscript's content.

Author contributions

LSM, PGY, MFL, SSD assisted in study concept and design of the studies. LSM collected and interpreted experimental data. VY performed the mass spectrometry analyses and provided technical support. JH collected experimental data related to cholesterol efflux and provided technical support. TP assisted in experimental data collection and analysis involving western blotting. MSB collected and interpreted data while MNO provided reagents and instrumentation support involving HAE experiments. WGJ performed data collection and analysis involving TEM measurements. LSM prepared the figures, drafted and revised the manuscript. VY, MSB, MNO, WGJ, PGY, MFL, and SSD assisted in analysis and interpretation of data, and provided critical reviews of the manuscript. MFL and SSD obtained project funding, provided technical and material support, supervised all aspects of the study, design, and execution. All authors reviewed the results and approved the final version of the manuscript.

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FIGURE 1.

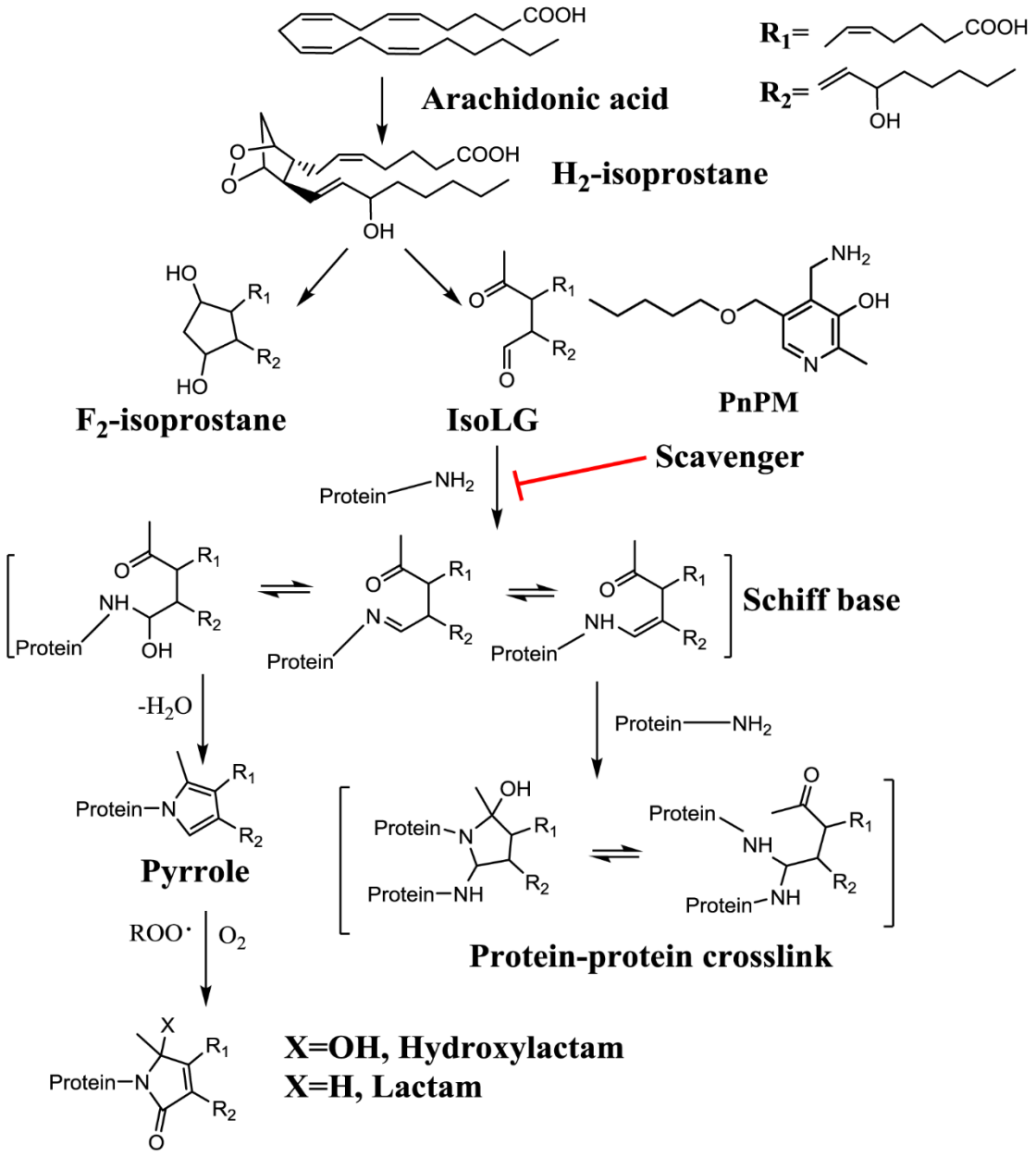
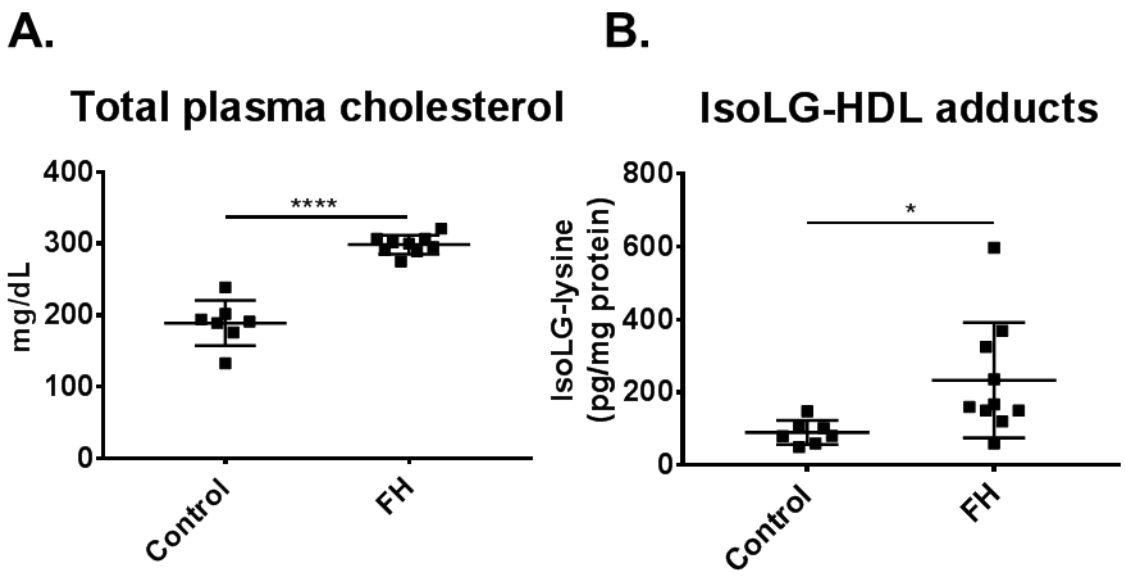


FIGURE 2.



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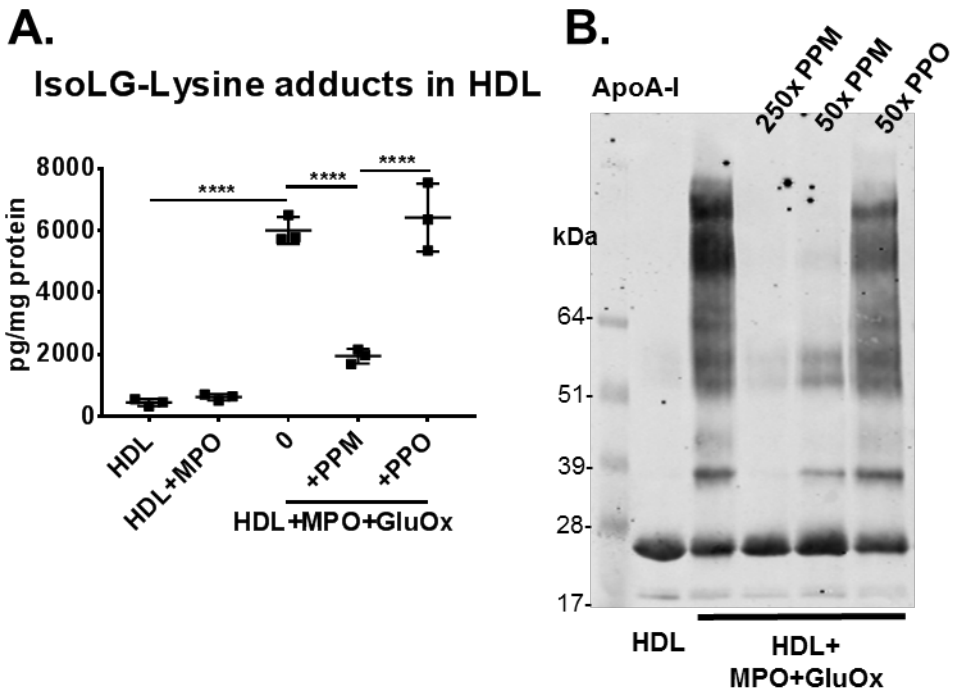

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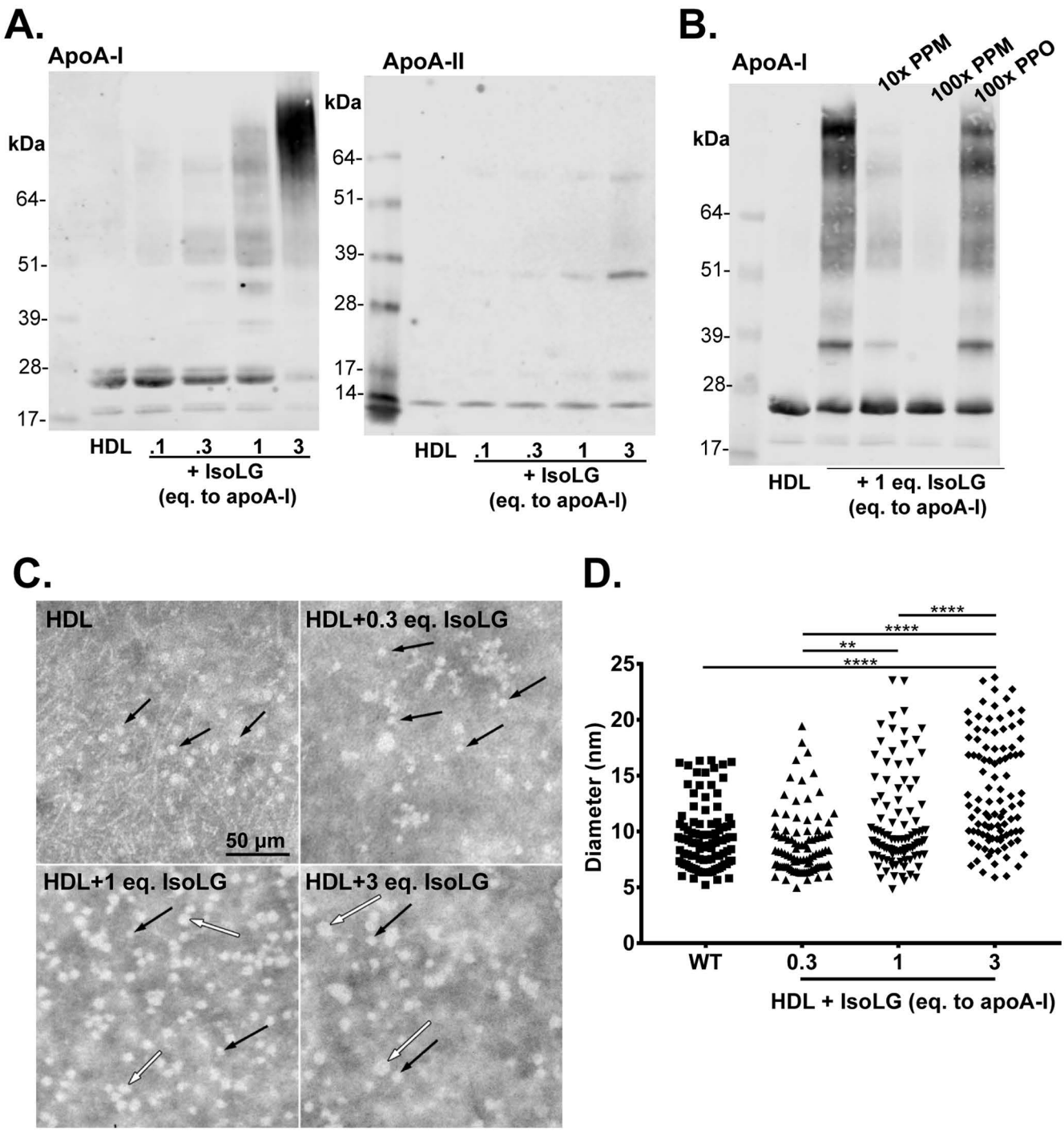
A.
ApoA-I
kDa
64-
51-
39-
28-
17-
HDL .1 .3 1 3
+ IsoLG
(eq. to apoA-I)
ApoA-II
kDa
64-
51-
39-
28-
17-
14-
HDL .1 .3 1 3
+ IsoLG
(eq. to apoA-I)
B.
ApoA-I
10x PPM
100x PPM
100x PPO
kDa
64-
51-
39-
28-
17-
HDL
+ 1 eq. IsoLG
(eq. to apoA-I)
C.
HDL
HDL+0.3 eq. IsoLG
50 µm
HDL+1 eq. IsoLG
HDL+3 eq. IsoLG
D.
****
****
**
****
Diameter (nm)
25
20
15
10
5
0
WT
0.3
1
3
HDL + IsoLG (eq. to apoA-I)

FIGURE 5.

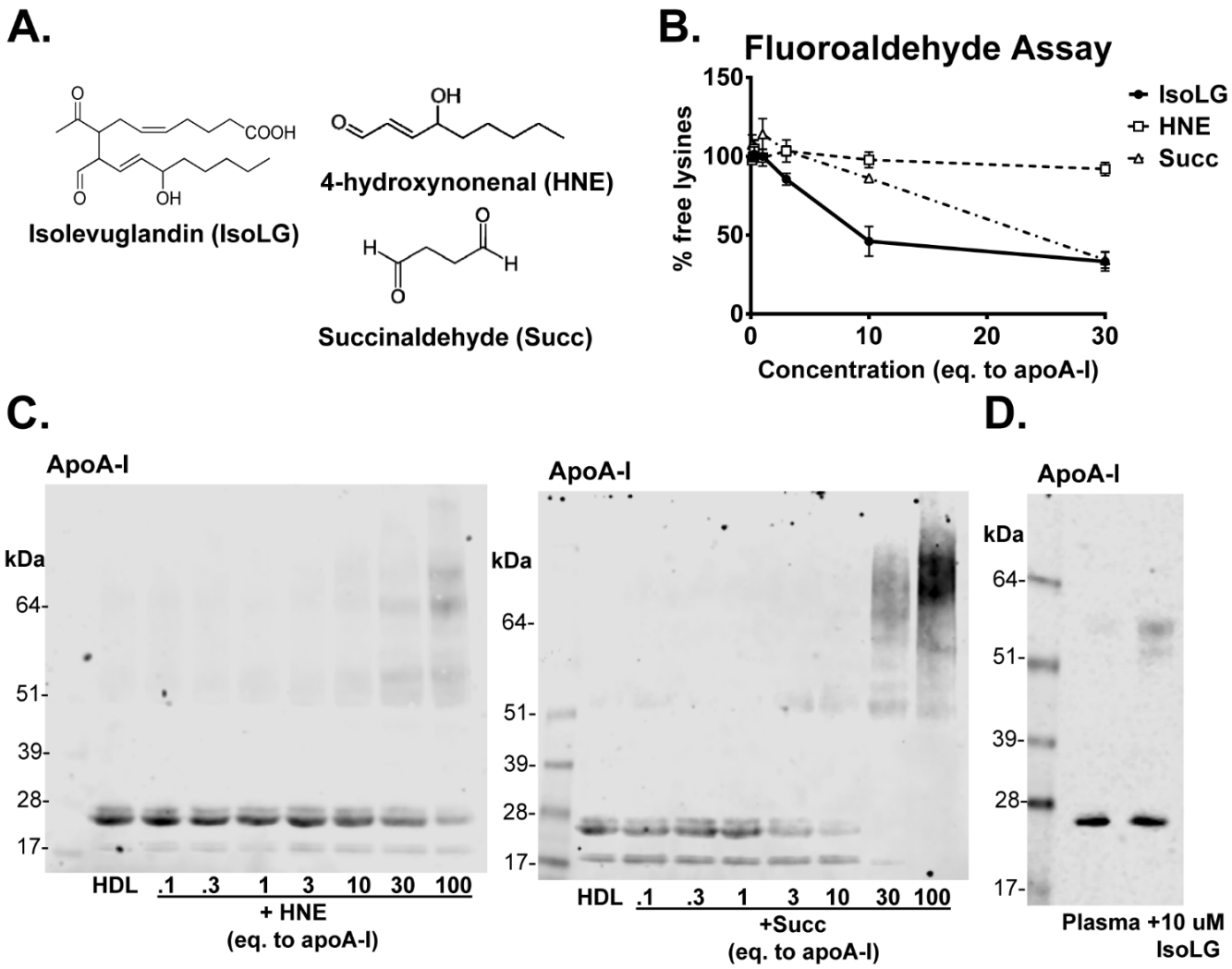

FIGURE 6.

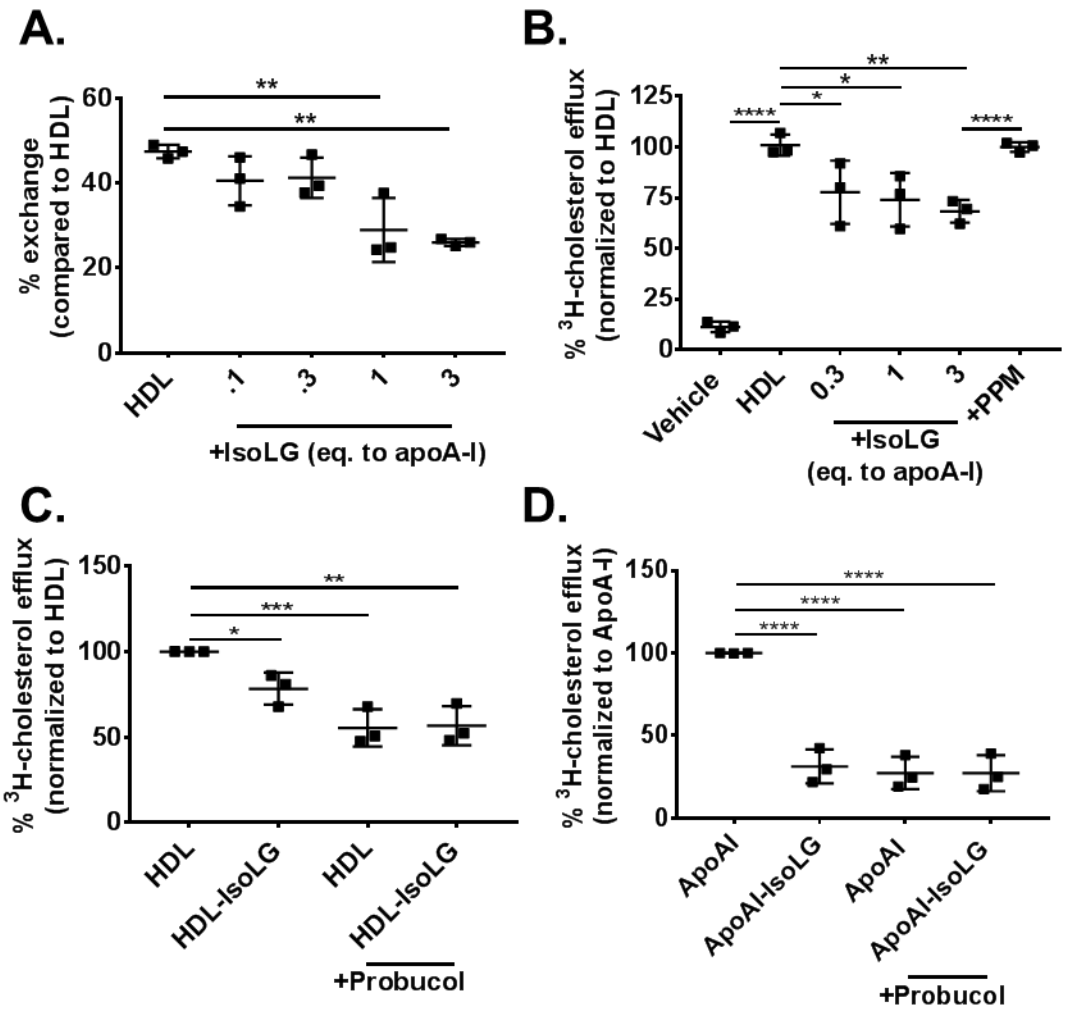

FIGURE 7.

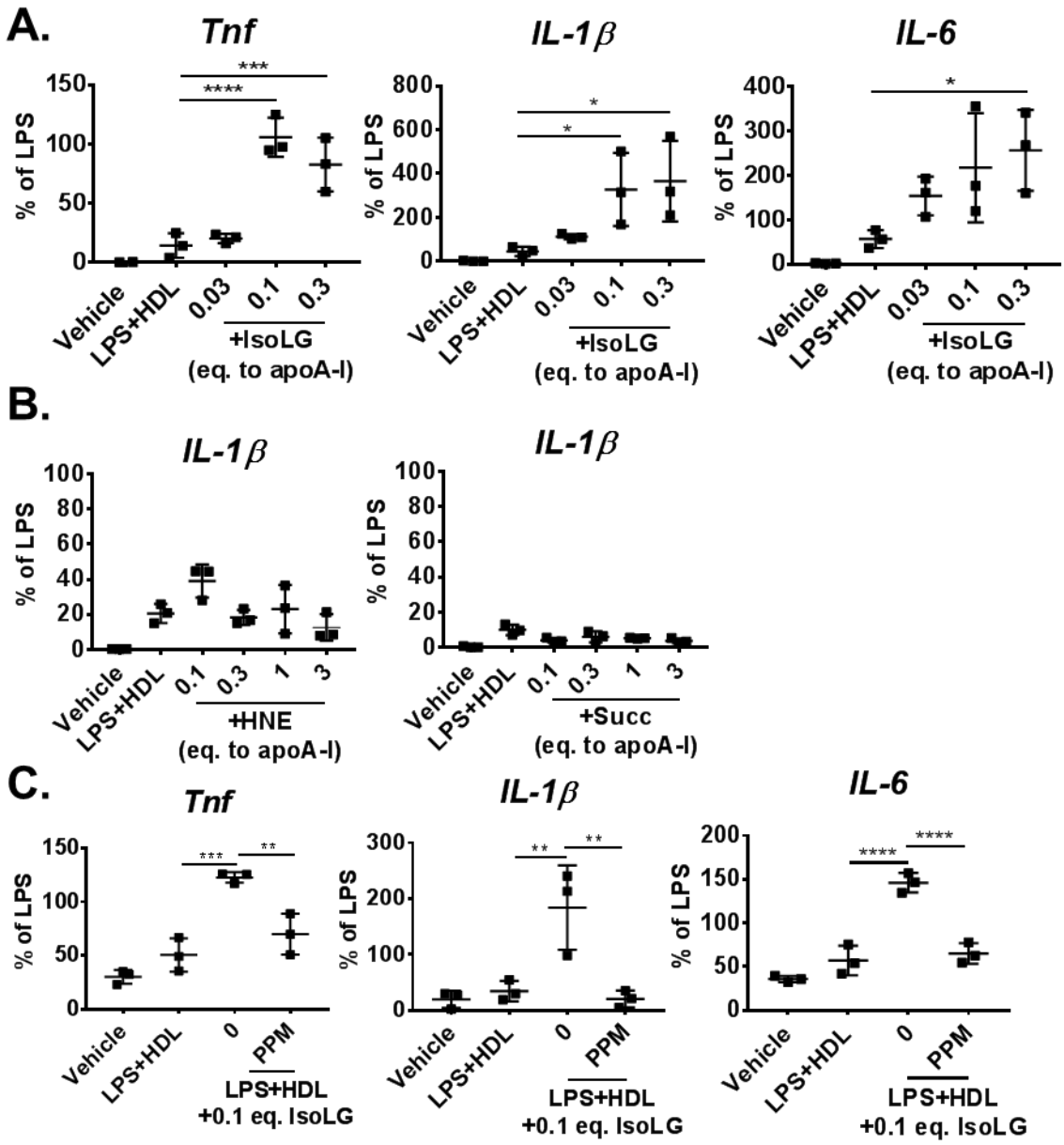

Figure Legends

Figure 1. Mechanism of IsoLG formation from peroxidation of arachidonic acid and its subsequent adduction and crosslinking of proteins.

Figure 2. IsoLG-protein adducts are increased in HDL isolated from plasma of patients with familial hypercholesterolemia (FH). Plasma was isolated from the blood of FH patients (n=10), 4 of which were heterozygous FH and 6 were homozygous FH prior to undergoing LDL apheresis. Control plasma was isolated from blood of healthy volunteers (n=7). HDL was isolated by DGUC. Levels of IsoLG-lysine adducts were determined by LC/MS/MS. Values are shown as mean ± SD. Statistical significance was calculated by Student's t-test. *P<0.1, ****P<0.0001.

Figure 3. (A) MPO oxidation of HDL generates IsoLG-lysine adducts, which are reduced with 1 mM PPM treatment but not with PPO. (B) MPO-oxidation of HDL crosslinks apoA-I which is reduced with PPM treatment but not PPO. HDL isolated from normal healthy subjects by DGUC was subjected to *ex vivo* oxidation by MPO and glucose oxidase/glucose/sodium nitrite system. Levels of IsoLG-lysine adducts were determined by LC/MS/MS. Protein crosslinking of apoA-I was determined by Western blot analysis. HDL was preincubated with PPM for 30 min before the addition of MPO and glucose oxidase/glucose/sodium nitrite. Experiments were performed three independent times. Results from individual experiments are plotted as mean ± SD. Western blots are representative of those from the three independent experiments.

Figure 4. IsoLG crosslinks HDL structural proteins apoA-I and apoA-II, resulting in HDL of larger size. HDL isolated from normal healthy subjects by FPLC was subjected to *ex vivo* modification of IsoLG. Crosslinking of HDL proteins (A) apoA-I and apoA-II by IsoLG, and (B) the prevention of crosslinking by PPM at 10x and 100x fold excess to IsoLG was determined by immunoblotting. PPO was used as an inactive analogue of PPM. Western blots are representative of experiments performed four times. (C) Modified HDL was subjected to negative staining and visualized using a transmission electron microscope. Black arrows denote small HDL particles while white arrows denote large spherical HDL particles. 200 HDL particles were sized and their distribution is depicted in (D). Statistical significance was calculated by one-way ANOVA with multiple comparisons. ***P<0.001, ****P<0.0001.

Figure 5. IsoLG is more reactive than other lipid aldehydes in adducting to lysine residues and crosslinking apoA-I in HDL. HDL isolated from normal healthy subjects by DGUC was subjected to *ex vivo* modification of various lipid aldehydes under aqueous conditions at 37ºC. Chemical structures of IsoLG, HNE, and succinylaldehyde (succ) (A). Extent of lysine adduction by various carbonyls (B) was determined by a fluoroaldehyde assay and shown as a % of free lysines left to adduct to OPA. Experiments were performed 3-4 times with replicate readings per sample. Values are represented as mean ± SD. (C) HNE and succinylaldehyde require at least 10 fold greater concentration to crosslink apoA-I in HDL when compared with IsoLG. (D) 10 μM IsoLG crosslinks apoA-I in human plasma. Plasma derived from healthy control human subjects was subjected to *ex vivo* modification of IsoLG. Crosslinking of apoA-I was determined by immunoblotting.

Figure 6. Modification of HDL by IsoLG inhibits the exchangeability of apoA-I on HDL and reduces ABCA1-mediated cholesterol efflux from apoE deficient murine macrophages. HDL isolated from normal healthy subjects by DGUC was subjected to *ex vivo* modification of IsoLG. (A) HDL-ApoA-I exchange was analyzed by electron paramagnetic resonance as described in Methods. Reactions were performed at a constant apoA-I concentration of 1 mg/ml. Experiments were performed three independent

times with triplicate readings of each sample. (B) Macrophage cholesterol efflux was assessed using **t**hioglycollate-induced macrophages harvested from the peritoneum of apoE deficient mice, and loaded with ^{3}H cholesterol and acetylated LDL. Vehicle denotes cell culture media with no HDL added to the cells. Efflux of ^{3}H to unmodified and modified HDL was calculated based on radioactive counts in the supernatant after 4 h and normalized to HDL control. PPM was preincubated with HDL for 30 min before addition of 3 eq. IsoLG. ABCA1-mediated cholesterol efflux was assessed similarly to the experimental design in (B) but with the addition of 10 uM Probucol treatment to the cells one hour before (C) HDL or (D) lipid-poor apoA-I treatment. Efflux experiments were performed three independent times with replicate wells per treatment. Results from individual experiments are plotted as mean ± SD. Statistical significance was calculated by one-way ANOVA with multiple comparisons compared to unmodified HDL (control). *P<0.05; **P<0.01; ***P<0.005; ****P<0.0001.

Figure 7. Modification of HDL by IsoLG induces a proinflammatory phenotype in murine macrophages. HDL isolated from normal healthy subjects by DGUC was subjected to *ex vivo* modification of IsoLG. Cells were treated (A) with LPS along with IsoLG modified HDL and (B) with LPS along with other lipid aldehydes modified HDL for 4 hours. (C) Effect of PPM on preventing IsoLG modification of HDL and macrophage inflammatory response. PPM was preincubated with HDL for 30 min before addition of IsoLG. Vehicle denotes cell culture media with no HDL or LPS added to the cells. Gene expression by qPCR was assessed after mRNA extraction. Experiments were performed independently three times with three wells per treatment. Results from individual experiments are plotted as mean ± SD. Statistical significance was determined by one-way ANOVA with multiple comparisons compared to LPS plus unmodified HDL (control). *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

Modification by isolevuglandins, highly reactive γ-ketoaldehydes, deleteriously alters HDL structure and function

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