Published in final edited form as:

Atherosclerosis. 2018 August ; 275: 333–341. doi:10.1016/j.atherosclerosis.2018.06.882.

Dietary 23-hydroxy ursolic acid protects against atherosclerosis and obesity by preventing dyslipidemia-induced monocyte priming and dysfunction

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Abstract

Background and aims—We demonstrated that dietary ursolic acid (UA) reduces atherosclerotic lesion size and improves kidney function in diabetic mice. Based on structure-function analyses of naturally occurring UA analogs, we synthesized 23-hydroxy ursolic acid (23-OHUA), a compound with structural features predicted to enhance its bioavailability and anti-atherogenic properties compared to UA. The goal of this study was to determine the anti-obesogenic and atheroprotective properties of 23-OHUA and its mechanism of action.

Methods—We performed chemotaxis assays to determine IC₅₀ of phytochemicals on primed THP-1 monocytes. We fed 12-week old female *LDLR*^{-/-} mice a high-fat diet (HFD) or a HFD supplemented with either 0.05% UA or 0.05% 23-OHUA, and measured monocyte priming, weight gain and atherosclerotic lesion size after 6 and 20 weeks.

Results—Both dietary UA and 23-OHUA prevented dyslipidemia-induced loss of MKP-1 activity, and hyper-chemotactic activity, a hallmark of blood monocytes priming and dysfunction, but they did not affect plasma lipids or blood glucose levels nor WBC and monocyte counts. After 20 weeks, mice fed 23-OHUA showed 11% less weight gain compared to HFD-fed control mice

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Conflicts of interests

RA is the President and CSO of Exprimon Corporation, a San Antonio based start-up company that plans to develop and sell nutraceuticals for the prevention of obesity and vascular diseases. The other authors have no conflicts of interest to declare.

Author contributions

H.N.N., Y.J.A. and E.A. M. performed experiments and contributed significantly in preparing the manuscript. R.A. designed the experiments and contributed significantly to the preparation of the manuscript.

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and a 40% reduction in atherosclerotic plaque size, whereas UA reduced lesion size by only 19% and did not reduce weight gain.

Conclusions—Dietary 23-OHUA reduces weight gain and attenuates atherogenesis in mice by protecting monocytes against metabolic stress-induced priming and dysfunction. Based on its mechanism of action, 23-OHUA may represent a novel therapeutic approach for the prevention and treatment of obesity and atherosclerosis.

Keywords

atherosclerosis; MKP-1; 23-hydroxy ursolic acid; monocytes; macrophages

1. Introduction

Monocyte adhesion, chemotaxis and the recruitment of monocyte-derived macrophages are key steps in the initiation and progression in atherosclerosis^{1, 2}. Metabolic stress caused by hyperglycemia and hyperlipidemia induces monocyte “priming” and dysfunction, resulting in accelerated chemotaxis in response to chemoattractants and the increased recruitment of macrophages to sites of inflammation^{3, 4}. Metabolic stress-induced priming of blood monocytes increases the formation of reactive oxygen species (ROS), resulting in, increased protein *S*-glutathionylation, the reversible formation of mixed disulfides between the cysteine of glutathione and redox-sensitive thiols (–SH) on protein cysteines, which can lead to protein inactivation^{5–7}. We showed that MAPK phosphatase 1 (MKP-1), a counter-regulator of the MAPK pathways that control monocyte adhesion and chemotaxis, is a key mediator of monocyte priming⁸. *S*-Glutathionylation of MKP-1 inactivates the enzymes and promotes its degradation, resulting in the hyperactivation of p38 and ERK pathways in response to chemokine stimulation, and thereby enhanced monocyte adhesion and chemotaxis and accelerated atherogenesis^{8, 9}.

We reported that ursolic acid (UA), a natural pentacyclic triterpenoid, protects THP-1 monocytic cells, murine peritoneal macrophages and human blood monocytes against priming and the hyper-responsiveness to chemoattractants by blocking metabolic stress-induced *S*-glutathionylation of actin and MKP-1, and by preserving MKP-1 activity¹⁰.

UA is a well-studied phytochemical found in herbs and the peels of fruits including apples and berries^{11, 12}. It has been reported to reduce a variety of cancers^{12–14}, increase brown fat¹⁵, reduce hyperlipidemia¹⁶, and improve diabetic complications¹⁷. We demonstrated that oleanolic acid, UA’s structural isomer, did not protect monocytes from metabolic stress-induced hypersensitization to chemoattractants as effectively as UA¹⁰. Interestingly, the only difference between the 2 isomers is in the position of one methyl group, suggesting UA’s unique structure confer its protective effects on monocytes.

Here we investigated the structure-function relationships between nine naturally occurring analogues of UA, which led to the identification of 23-hydroxy ursolic acid (23-OHUA) as a potential therapeutic phytochemical for the prevention and treatment of atherogenesis. We found that the addition of a hydroxyl group on carbon 23 of UA did not affect UA’s protective properties against monocyte priming but enhanced its solubility and, therefore,

potentially its bioavailability when administered as a dietary supplement. Our data also indicate that UA and 23-OHUA may act through a similar pathway and that 23-OHUA, like UA, may be atheroprotective. 23-OHUA has been extracted from various medicinal herbs and studied for its anti-cancer effects^{18, 19}, but its therapeutic properties in the context of metabolic diseases and chronic inflammatory diseases are unknown. We, therefore, examined whether 23-OHUA at physiological relevant doses protects mice against atherosclerosis and its mechanism of action.

2. Materials and methods

2.1. Cell culture and monocyte priming

THP-1 cells (ATCC) were cultured in “THP-1 medium” (RPMI 1640, 5 mM glucose, 10% fetal bovine serum (FBS), 2% Glutamax, 1% penicillin/streptomycin, 1% HEPES, 0.1% β -mercaptoethanol). Human blood was obtained from the South Texas Blood and Tissue Center. Mononuclear cells were isolated from human blood using density gradient centrifugation over Ficoll. Primary human monocytes were further purified with magnetic beads (EasySep, StemCell) according to the manufacturer’s suggestions; monocyte purity was 90–95%. Primary monocytes were centrifuged for 5 min at 1200 rpm (400g) and at 25°C, resuspended in THP-1 medium at 0.5×10^6 cells/ml, and cultured for 48 h at 37°C and 5% CO₂.

For chemotaxis and monocyte priming assays, THP-1 cells (0.75×10^6 /ml) or purified human blood monocytes (1.0×10^6 /ml) were incubated for 1 h with either vehicle or phytochemicals (0.03 μ M to 3 μ M) before being metabolically primed by adding freshly isolated human LDL (100 μ g/ml) plus D-glucose (20 mM) to the culture medium and incubating the cells for an additional 24 h. Phytochemicals were solubilized in dimethylsulfoxide (DMSO; < 0.01% v/v).

2.2. LDL isolation

LDL was isolated from pooled human plasma obtained from healthy donors. Human plasma was mixed with KBr and aliquoted into tubes (Beckman, 13 $\times$ 56 mm). Plasma and KBr solution was overlaid with 0.15 M NaCl and centrifuged (Beckman Coulter Optima MAX-XP, TLA-110 rotor, 90,000 rpm, 25 minutes, 10°C) as described previously^{20, 21}. The LDL band was then isolated and added to an additional 0.15 M NaCl, and spun again (87,000 rpm, 80 minutes, 10°C). The upper band containing LDL was isolated and passed through a Zeba Desalting Column (Pierce). LDL was sterile filtered (0.45 μ m sterile filter, MILLEX-HV) and stored at 4°C.

2.3. Chemotaxis assay

After culturing unprimed and primed monocytes in the absence or presence of phytochemicals, cells were collected and centrifuged at 1200 rpm (300 $\times$ g), 5 min at 25°C. Cells were then resuspended in THP-1 medium with 0.1% FBS and Trypan Blue and counted using an automated cell counter (Cellometer Vision, Nexcelcom Biosciences). Cell concentrations were adjusted to 0.75×10^6 cells/ml to normalize concentrations for all treatments. Solubilized MCP-1 was added to THP-1 medium to a final concentration of 1

nM MCP-1 (“chemoattractant medium”). Migration was assessed using a 48-well modified Boyden chamber (Neuro Probe). Chemoattractant medium was loaded in the bottom wells (25 μ l) with a polycarbonate membrane with 5 μ m pores (Neuro Probe) separating the two chambers. An equal volume of cells was loaded into the wells of the top chamber, and the chamber was placed in the incubator for 1.5 h at 37°C and 5% CO₂. Cells that had adhered to the top of the polycarbonate membrane were scraped off with a filter wiper, and the membranes were then briefly washed in warm PBS. The membrane was then fixed in methanol for 1 minute, air-dried, and stained with propidium iodide (PI, 1 μ M) for 30 min. Excess PI was then removed by washing the membrane in sterile water for 30 minutes. Membranes were blotted and fluorescence was measured using a KODAK Image Station 4000MM (Carestream). Cell number correlated with net intensity of spots on membranes. The PI stain method was validated against a manual counting method¹⁷ using Diff-Quik. Briefly, membranes were stained with Dif-Quik and five fields of view were counted.

2.4. In vivo chemotaxis assay

Matrigel plug assays were used to determine monocyte chemotaxis in response to chemoattractant (MCP-1) *in vivo* both at 6 and 20 weeks as we described previously⁸. Briefly, Matrigel (BD Biosciences) supplemented with vehicle (injected in mouse’s left flank) or MCP-1 (300 nM, injected in mouse’s right flank) was injected subcutaneously. After 3 days, mice were sacrificed and the plugs removed. Plugs are dissolved in disperse for 2 hours at 37°C. Monocytes were washed with PBS and counted using an automated cell counter (Cellometer Vision, Nexcelcom Biosciences). The number of monocytes within plugs were counted following digestion.

2.5. MKP-1 activity assay

The MKP-1 activity assay was performed as described previously⁸. Briefly, phosphatase release from a phospho-tyrosine peptide (PTP) substrate when incubated with cell lysates in the presence or absence of 40 μ M sanguinarine, a specific inhibitor of MKP-1²² was measured. Assays were initiated by adding 100 μ M of PTP substrate (in ddH₂O) to cell extracts (2 μ g protein) diluted in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40 and warmed to 30 °C. Phosphate cleavage from the PTP substrate release was assayed spectrophotometrically as the amount of inorganic phosphate released using a VersaMax reader (Molecular Devices) – KH₂PO₄ was used as standard. Sanguinarine-sensitive phosphate release from the PTP substrate was attributed to MKP-1.

2.6. Animals

Female *LDL-R*^{-/-} mice (B6.129S7-LdlrtmIHer/J, stock no. 002207) on a C57BL/6J background were obtained from The Jackson Laboratories (Bar Harbor, ME). Mice were housed in colony cages and maintained on a 12-h light/12-h dark cycle. All studies were performed with the approval of the UT Health San Antonio Institutional Animal Care and Use Committee. Forty-eight mice aged 12 weeks were randomized into 6 groups, 8 mice per group: maintenance diet (MD) for 6 weeks; high fat diet (HFD; 21% fat wt/wt, 0.15 cholesterol wt/wt; AIN-76A, Bio-Serv) or HFD + 0.05% 23-OHUA or HFD + 0.05% 23-OHUA for 6 weeks; or HFD, HFD + 0.05% 23-OHUA or HFD + 0.05% 23-OHUA for 20 weeks. Phytonutrient-supplemented diets were prepared by Bio-Serv. Mice were sacrificed

at 6 weeks to characterize monocyte priming and the onset of atherosclerosis, and after 20 weeks of diet challenge to assess the progression of atherosclerosis. (Power = 0.8, significance = 0.05, standard deviation = 20%, and an expected difference of means = 50% reduction; SigmaPlot software). Fasted bodyweights were assessed weekly.

2.7. Blood and body composition analysis

We obtained blood from mouse tail veins and measured overnight-fasted blood glucose biweekly with a Contour glucometer (Contour; Bayer). In addition, glucose tolerance tests were performed before sacrifice to determine insulin resistance (Supplemental Figure 3). Plasma cholesterol and triglyceride levels were determined with enzymatic assay kits (Wako Chemicals) using blood obtained by cardiac puncture. Liver toxicity was assessed by measuring AST and ALT activity in plasma using enzymatic activity assay kits (Abcam). We used dual-energy X-ray absorptiometry (DEXA) analysis to assess fat mass and lean body mass before each sacrifice.

2.8. Atherosclerotic lesion measurement and characterization

Atherosclerotic lesions in the descending aorta and aortic root from mice in each group were quantified at both the 6-week and the 20-week time-points as described previously²³. Tissues were stored in 4% paraformaldehyde until imaged with a Spot digital camera (Leica) mounted on a dissecting microscope and quantification was completed with ImagePro software. The aortic roots were placed in OCT, flash frozen by liquid nitrogen, and stored at -80°C for further analysis. Tissue blocks were placed in a cryostat and sectioned as previously described¹⁷. Briefly, 10 μm sections were cut, stained, and analyzed according to protocols established. We used Oil Red O (ORO, Abcam) stains to characterize sizes of lesions and CD68 (Bio-Rad, clone FA-11) stains to assess macrophage infiltration.

2.9. Statistical analysis

Calculations and statistical tests were completed with SigmaPlot 14 software. Data were tested for use of parametric or nonparametric post hoc analysis, Tukey method. All data are presented as mean $\pm$ SE of at least 3 independent experiments. Results were considered statistically significant at the $p < 0.05$ level.

3. Results

3.1. 23-OHUA and UA protect THP-1 monocytes from metabolic stress-induced dysfunction

To identify novel and more potent anti-atherogenic compounds with the same unique mechanism of action as UA^{10, 17}, we first investigated the structure-function relationships between 9 naturally occurring analogues of UA. Previously, we showed that oleanolic acid (OA) is 3.5-fold more potent than UA in blocking metabolic priming of monocytes¹⁰, despite only differing in the position of a single methyl group at carbon 29 of UA (Fig 1A) or carbon 30 of OA (Fig 1D). To further examine the effect of moving the methyl group in position 29 (Fig. 1, solid red circle) to position 30 (Fig. 1, dashed red circles), and to identify additional structural features of UA that confer its ability to protect monocytes against metabolic stress-induced dysfunction, we used a multi-chamber chemotaxis assay to assess the ability of UA and nine naturally occurring UA analogues to inhibit metabolic priming of

THP-1 monocytes (Fig. 2). Metabolic stress was induced – in either the presence or absence of phytochemicals - by exposing the cells for 24 h with freshly isolated human LDL (100 µg/ml) and high concentrations of glucose (25 mM), conditions that mimic monocyte priming and dysfunction observed *in vivo*^{3, 4, 8, 10}. The nine naturally occurring analogues of UA were selected based on their close structural relationship to UA and included: uvaol (UV) and its isomer erythrodiol (ED), α-amyrin (α-A) and its isomer β-amyrin (β-A), corosolic acid (CA), asiatic acid¹⁸, madecassic acid (MA) and hederagenin (HA) (Fig 1). In addition to examining the role of the methyl at position 29/30 (Fig 1, **red circles**), these structural analogues allowed us to investigate whether increasing hydrophobicity at position 17 (Fig. 1, **blue circles**) or decreasing hydrophobicity in ring A (Fig. 1, **green circles**) would alter the protective effects of the phytochemical on monocyte priming.

In agreement with our previous data, OA was significantly less potent (Fig. 2D, IC₅₀=1.56 µM) than UA (Fig. 2A, IC₅₀=0.1 µM) at blocking monocyte priming. To determine whether the dimethyl group on position 30 of other UA analogues reduced efficacy in blocking monocyte priming, we compared uvaol (Fig. 1B, dimethyl at C-30) with erythrodiol (Fig. 1E, methyl at C-29). Indeed, the protective activity of uvaol (Fig. 2B, IC₅₀=2.4 µM) was completely lost in its isomer, erythrodiol (Fig. 1E and 2E). Neither α-amyrin (Fig. 1C) nor β-amyrin (Fig. 1F) blocked monocyte priming at doses below 3 µM (Fig. 2C and 2F), but interestingly, both compounds actually enhanced monocyte priming, α-amyrin to a greater degree than β-amyrin. These data indicate that the dimethyl group on position 30 of OA and other UA analogues dramatically reduces their efficacy in blocking monocyte priming.

To determine how increased hydrophobicity at C-17 affects the potency of UA analogues in blocking monocyte priming, we compared UA with uvaol and α-amyrin (Fig. 1A and 1B, **blue circles**). UA (IC₅₀=0.1 µM) was more potent than uvaol (IC₅₀ = 2.4 µM) at blocking THP-1 priming, and uvaol was more potent than α-amyrin (IC₅₀> 3 µM) (Fig. 2). Similarly, OA (Fig. 1D), which is less hydrophobic at C-17 than erythrodiol (Fig. 1E), was more effective in blocking monocyte priming than erythrodiol (Fig. 2D and 2E). These data indicate that increasing hydrophobicity at C-17 also reduces potency of these phytochemicals in blocking monocyte priming.

Lastly, the effect of hydrophobicity of the A ring was assessed by comparing UA (Fig. 1A) with corosolic acid (Fig. 1G), asiatic acid (Fig. 1H), and madecassic acid (Fig. 1I, **green circles**). Again, UA (IC₅₀=0.1 µM) was more effective than corosolic acid (IC₅₀=2.1 µM) in blocking monocyte priming, and neither asiatic acid nor madecassic acid blocked monocyte priming at doses than 3 µM (Fig. 2). These data suggest that decreasing the hydrophobicity of the A ring reduces the efficacy of these compounds in blocking monocyte priming. To corroborate these findings, we compared OA (Fig. 1D) with hederagenin, which possesses one additional hydroxyl group on C-23 (Fig. 1J). Surprisingly, hederagenin (IC₅₀=0.51 µM) was more potent in blocking monocyte priming than oleanolic acid (IC₅₀=1.56 µM) (Fig. 2J versus 2D), suggesting that an additional hydroxyl group at position 23 enhances the efficacy of this phytochemical class in blocking monocyte dysfunction. We tested this hypothesis by comparing custom-synthesized 23-hydroxy ursolic acid (23-OHUA, Fig. 1K) with UA (Fig. 1A). 23-OHUA (IC₅₀=0.3 µM) was more potent than the other naturally occurring UA analogues tested and approximately equally potent as UA in protecting THP-1

monocytes from metabolic priming (Fig 2K versus 2A). Of note, none of the compounds tested were toxic below 30 μM (Supplemental Figure 1). Importantly, at 3 μM , both UA and 23-OHUA also completely blocked accelerated chemotaxis induced by metabolic priming in purified human blood monocytes (Supplemental Figure 2, dark bars), but neither UA nor 23-OHUA affected normal MCP-1-induced cell migration (Supplemental Figure 2, light bars).

3.2. Dietary 23-OHUA slows weight gain in HFD-fed *LDLR*^{-/-} mice

Next, we wanted to examine and compare the putative monocyte protective properties of 23-OHUA and UA in an established mouse model of atherosclerosis. To this end, we fed 24 female *LDLR*^{-/-} mice either a HFD or a HFD supplemented with either 0.05% w/w of 23-OH UA or 0.05% w/w UA. For 20 weeks, we measured weekly body weight and bi-weekly fasted blood glucose levels. At the end of 6 weeks, we sacrificed 8 mice in each diet group mice. At that point, no statistically significant differences in body weights (Fig. 3) or body composition as determined by DEXA were observed (Supplemental Fig. 3A). However, after 6 weeks, body weights of the three diet groups began to segregate, with mice on a HFD consistently gaining more weight than mice on either the 23-OHUA or the UA supplemented diet. After 20 weeks of diet feeding, mice on the 23-OHUA-supplemented diets gained 11.4% less weight than the HFD-only group ($P=0.002$). UA-supplemented mice, on average also gained less weight than HFD-fed mice (6.3%), but the difference in weight gain did not reach statistical significance ($P=0.23$). While the differences in fat and lean body mass measured by DEXA after 20 weeks of diet feeding were still not statistically significant, between the three groups, the data suggests that the reduced body weight in the 23 OHUA-fed group was mainly due to reduced body fat (Supplemental Fig. 3B). However, the reduction in weight gain achieved by 23-OHUA did not (yet) significantly improve glucose tolerance (Supplemental Fig. 3C+D)

After 6 weeks of diet feeding, plasma levels of 23-OH UA and UA were 0.037 μM and 0.032 μM , respectively, and, after 20 weeks, increase to 0.52 μM in 23-OHUA-fed mice and 0.4 μM in UA-fed mice (Supplemental Fig. 4). UA-fed mice also showed low levels of 23-OHUA (< 50 nM) in their plasma. After 20 weeks, plasma concentration of 23-OH UA reached 0.52 μM . Although the difference in plasma levels in 23-OHUA and UA between the two groups after 20 weeks of diet feeding did not reach statistical significance, the 28% higher plasma levels of 23-OHUA versus UA suggest a better bioavailability for 23-OH UA than UA. The slightly higher plasma levels may account for the better protection against weight gain in 23-OHUA-fed mice.

Neither UA nor 23-OHUA supplementation affected fasted blood glucose, plasma lipid levels (Table 1) or lipoprotein profiles (Supplemental Fig. 5A and B). RBC, WBC and platelet counts were also not altered by either of the phytochemicals (Table 1). Finally, we did not observe any liver toxicity by either of the two compounds in this mouse model of atherosclerosis at either time point (Supplemental Fig. 6).

3.3. Dietary supplementation with 23-OHUA preserves MKP-1 activity and reduces macrophage recruitment in *LDLR*^{-/-} mice in vivo

To test whether 23-OHUA and UA protected blood monocytes against metabolic stress-induced priming and dysregulation, we injected MCP-1 and vehicle-loaded Matrigel plugs in the left and right abdomen of *LDLR*^{-/-} mice. We showed previously that HFD-feeding induces a 3–4-fold increase in macrophage recruitment into MCP-1-loaded Matrigel plugs. In this study, as early as 6 weeks after initiating HFD feeding, dietary 23-OHUA reduced MCP-1-induced recruitment of monocyte-derived macrophages in HFD-fed mice by 27%, suggesting that 23-OHUA protects blood monocytes against metabolic priming (Fig. 4A). The protective effect of 23-OHUA was maintained at 20 weeks, (Fig 4B). UA also reduce macrophage recruitment in response to MCP-1, but the decreases did not reach statistical significance at either time point (Fig. 4).

Metabolic stress induces the inactivation and subsequent degradation of MKP-1, a key counter-regulator of the MAPK pathways controlling adhesion and chemotaxis of monocytes^{8,9}. HFD-feeding for only 6 weeks reduced MKP-1 activity in freshly isolated blood monocytes by 42% (Fig. 4C), a biomarker of metabolic stress-induced monocyte priming and dysfunction^{8,9}. After 20 weeks, MKP-1 activity in blood monocytes was reduced by 49% (Fig. 4D). 23-OHUA, and to a lesser extent UA, rescued MKP-1 activity in blood monocytes (Fig. 4C and D), strongly suggesting that both compounds protect monocytes against metabolic priming and their conversion into a hyper-chemotactic pro-atherogenesis phenotype. Since monocyte priming correlates with and contributes to atherogenesis in mice^{4,8,9}, these findings strongly suggest that 23-OHUA and UA protect against atherogenesis and the development of atherosclerotic plaques.

3.4. 23-OH UA supplementation reduces atherosclerotic lesion development and macrophage recruitment into lesions

To test this hypothesis, we measured atherosclerotic lesion sizes in two different vascular beds at both time points in all three groups of mice. *En face* analysis of the aortic arch and entire descending aorta revealed only a few small lesion after 6 weeks of HFD feeding, and we observed no significant differences between groups (Fig. 5A). At 20 weeks, aortic lesions covered over 17% of the entire aorta in the HFD-fed mice (Fig. 5B and E). Compared to HFD-fed mice, 23-OHUA-supplemented mice showed a 38% reduction in plaque size, whereas UA reduced lesion size by only 11%, demonstrating that 23-OH UA is significantly more potent in attenuating atherosclerosis. Analysis of the ORO-stained aortic root sections showed similar findings (Fig. 5C and F), thereby underscoring 23-OHUA's greater atheroprotective effects compared with UA (Fig. 5C). Macrophage content in aortic root lesions was also significantly reduced of mice treated with 23-OH UA (52%) or UA (41%) compared with the HFD only-fed group, however, the reduction in UA-fed mice did not reach statistical significance (Fig. 5D and F).

4. Discussion

In this study, we show that dietary 23-OHUA is a potent inhibitor of atherogenesis in HFD-fed *LDLR*^{-/-} mice and protects these mice against HFD-induced weight gain. 23-OHUA

was more potent than its parent compound UA in protecting against both atherosclerosis and weight gain. However, both compounds appear to exert their protective effects through the same, novel mechanism, i.e. by protecting blood monocytes against metabolic stress-induced priming, dysfunction and reprogramming^{4, 8, 9, 24} rather than lipid or glucose lowering. 23-OHUA's and UA's effects are at least partially mediated through the ability of both compounds to protect MKP-1, a central regulator of monocyte recruitment and macrophage functions^{8, 9, 25}, from oxidative inactivation and subsequent degradation induced by metabolic stress.

23-OHUA extracted from *Lagerstroemia speciosa* and *Juglans sinensis*¹⁹, has been shown to be an effective anticancer compound^{18, 26}, but data on its potential anti-inflammatory properties is limited. With the exception of an additional hydroxyl group in position 23, 23-OHUA has the same structure as UA, which explains why 23-OHUA appears to function through the same molecular mechanisms as UA. Indeed, our demonstration that both UA and 23-OHUA block metabolic stress-induced MKP-1 inactivation in monocytes support this notion. 23-OHUA, like UA, is non-toxic at physiologically relevant concentrations, and far more effective at inhibiting monocyte priming than the other analogues of UA we examined. 23-OHUA's predicted solubility is greater than that of UA (as predicted by ChemAxon, data; not shown), which may account for the slightly higher plasma levels, and 23-OHUA is, at least in cell culture, equally effective as UA in protecting monocytes from metabolic dysfunction. The exact molecular target(s) of these two analogues in blocking monocyte priming is unknown. One possible mechanism through which both compounds act may involve a reduction in the flux of reactive oxygen species (ROS) in monocytes, which is required for the oxidation, *S*-glutathionylation, and inactivation of MKP-1⁹. Indeed, we have reported that UA can reduce total protein-*S*-glutathionylation¹⁰. However, the chemical structure of 23-OHUA and UA indicates that these compounds are not easily oxidized and thus poor scavengers of ROS. It is therefore more likely, that both 23-OHUA and UA prevent protein thiol oxidation by either targeting signaling pathways that lead to ROS production or directly inhibiting the source of ROS generation, e.g. NADPH oxidase or mitochondria.

We reported that in a mouse model of diabetic atherosclerosis, supplementing the diet with 0.2% UA prevents atherosclerosis, lowers blood glucose and improves survival¹⁷. We also demonstrated that monocyte priming and dysfunction induced by metabolic disorders contribute to and accelerate atherosclerotic lesion formation in mice⁸ and that UA prevents monocyte priming^{10, 17}. In this study we now show that at a significantly lower dietary dose of 0.05%, which for UA corresponds to approximately 8 apples per day for humans¹⁷, both 23-OHUA and UA blocked monocyte dysfunction and prevented atherosclerosis in HFD-fed mice. The higher apparent efficacy of 23-OHUA versus UA in prevented both monocyte dysfunction and atherosclerosis is difficult to explain solely by 23-OHUA's higher water solubility and its slightly higher plasma levels. It is likely that dietary 23-OHUA may have additional, monocyte-independent benefits. Indeed UA has been reported to protect the endothelium against inflammatory activation and dysfunction^{27, 28}. Thus, protecting the endothelium against metabolic stress-induced injury and dysfunction may contribute to 23-OHUA's anti-obesogenic and atheroprotective properties. UA also modulate T-helper type 1

(Th1) and T-helper type 2 (Th2) cytokine production, which may affect proatherogenic T-cell responses^{29, 30}.

Surprisingly, we found that 23-OHUA also has a strong anti-obesogenic effect. The mechanisms underlying the anti-obesogenic property of 23-OHUA are not clear at this time. We did not assess food intake, which could account for the reduced weight gain. Alternatively, 23-OHUA may be increasing the metabolic rates of these mice. For example, UA has been shown to stimulate skeletal muscle Akt activity¹⁵, which is associated with an increase in energy expenditure. 23-OHUA may be functioning in the same way. Kunkel et al. also reported that UA enhances glucose tolerance and lowers blood glucose in HFD-fed C57BL/6 mice¹⁵. In our model, neither UA nor 23-OHUA affected blood glucose or lipid and lipoprotein levels. However, we administered a 3-fold lower, but physiologically more relevant doses of both compounds compared with that study. As an alternative mechanism, we propose that both the decrease in atherosclerosis and reduced weight gain are mediated by the protective effects of 23-OHUA on metabolic stress-induced monocyte priming and dysfunction, resulting in reduced recruitment of monocyte-derived macrophages into tissues, including adipose tissues and the aorta. We demonstrate that monocyte dysfunction in HFD-fed mice occurs as early as 6 weeks as evidenced by the increased recruitment of monocyte-derived macrophages into MCP-1-loaded Matrigel plugs implanted in these mice. Monocyte MKP-1 activity, a marker of monocyte priming, was restored to similar levels in both UA and 23-OHUA-fed mice, however, 23-OHUA showed a significantly more pronounced effect on both weight gain and atherosclerosis lesion development. This suggests that in addition to preventing monocyte priming, 23-OHUA may also exert direct anti-inflammatory effects on the adipose tissue, the vasculature and possible other organs.

In summary, with 23-OHUA, we identified a novel, potent anti-obesogenic and atheroprotective phytochemical, that at least in part exerts its effects by suppressing monocyte priming and dysfunction. 23-OHUA may therefore be an excellent candidate for a dietary supplement and/or adjunct therapy for the prevention of atherosclerosis, obesity and possibly other metabolic diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support

This project was supported by grants to R.A. from the NIH (AT006885) and to H.N.N. from the American Heart Association predoctoral fellowship (16PRE29820000).

The authors wish to thank Javier Esparza and the Histology Core Facility at UT Health San Antonio for technical assistance on the manuscript.

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Highlights

Dietary 23-hydroxy ursolic acid reduces weight gain in atherosclerosis-prone mice fed a high-fat diet by 11%.

Dietary 23-hydroxy ursolic acid reduces atherosclerotic plaque size in atherosclerosis-prone mice fed a high-fat diet by 40% without affecting plasma lipid, lipoprotein or glucose levels.

23-Hydroxy ursolic acid prevents monocyte priming and dysfunction induced by dyslipidemia and hyperglycemia, which may account for its anti-obesogenic and atheroprotective properties.

Figure 1. Schematic showing 10 naturally occurring structural analogues of ursolic acid
(A – C) Molecular structure of UA and UA analogues with altered hydrophobicity at C-17 (uvaol and α-amyrin). **(D – F)** Molecular structure of oleanolic acid (OA) and OA analogues with bimethyl groups (erythrodiol and β-amyrin). **(G – I)** Molecular structure of UA analogues with additional hydroxyl groups (corosolic acid, asiatic acid, and madecassic acid) in the A-ring. **(J + K)** Molecular structure of hederagenin, an analog of OA with an additional hydroxyl at C-23 and the structure of 23-hydroxyl ursolic acid (23-OHUA). Red circles show variations in methylation at the C-29 versus C-30 position. Blue circles indicate changes in polarity at the C-17 position, and green circles indicate changes at the A-ring region. Solid circles indicate identical structural features as UA, and dashed circles indicated altered residues when compared with UA.

Figure 2. Only ursolic acid and 23-hydroxy ursolic acid are potent inhibitors of blood monocyte priming and dysfunction

Chemotaxis assays using control and metabolically primed human blood monocytes were conducted as described under “Material and Methods”. Metabolic priming was induced in the presence of 0 – 3 μM (A – C) ursolic acid, uvaol or α -amyrin, (D – F) oleanolic acid, erythrodil or β -amyrin, (H – I) corosolic acid, asiatic acid or madecassic acid, (J + K) hederagenin or 23-OH ursolic acid (23-OHUA). Accelerated chemotaxis is defined as the change in MCP-1-induced chemotaxis over 1.5 h in metabolically primed blood monocytes (HG+LDL treated, 100% acceleration) versus unprimed control blood (0% acceleration). IC_{50} values for each phytochemical are shown in the upper right corner of each graph, and were determined using SigmaPlot software ($n=4-5$).

Figure 3. 23-OHUA reduces HFD-induced weight gain in atherosclerosis-prone *LDLR*^{-/-} mice *LDLR*^{-/-} mice were fed a high-fat diet (HFD, black circles) or HFD supplemented with either 0.05% w/w ursolic acid (UA, dark gray circles) or 0.05% w/w 23-OHUA (light gray circles). #: *P*<0.05, *: *P*<0.001. (n=8). Weights were monitored weekly (A) and weight gain over the 20-week period was calculated for each animal as the area under the curve (AUC; B).

Figure 4. 23-OHUA prevents monocyte priming and restores monocyte MKP-1 activity *in vivo* (A). Quantification of macrophages recruited into MCP-1-loaded Matrigel plugs implanted into *LDLR*^{-/-} mice fed a HFD for 6 weeks or (B) 20 weeks. (C) MKP-1 activity in blood monocytes isolated from mice fed either a maintenance diet (MD) or a HFD or a HFD supplemented with 0.05% w/w UA or 0.05% w/w 23-OHUA supplemented mice for 6 weeks. At 6 weeks, maintenance diet fed mice had an average of 70 pmol/ug of MKP-1 (data not shown). (D) MKP-1 activity of mouse blood monocytes at 20 weeks. (n=8).

Figure 5. 23-OHUA protects $LDLR^{-/-}$ mice against atherosclerosis

$LDLR^{-/-}$ mice were fed a high-fat diet (HFD) or a HFD supplemented with either 0.05% w/w ursolic acid (UA) or 0.05% w/w 23-OH UA for 6 weeks and 20 weeks. (A) Lesion size determined *en face* analysis of mouse aortas after 6 weeks and (B) 20 weeks on a HFD. (C) Lesion size determined in ORO-stained aortic root sections after 20 weeks of HFD feeding. (D) Macrophage content quantified by anti-CD68 antibody-based IHC in aortic root lesions after 20 weeks of HFD feeding. (E) Representative images of ORO-stained mouse aortas. (F) Representative images of ORO-stained and of anti-CD68 antibody-based IHC stained aortic root sections. (n=8).

Table 1

Biological parameters for mice fed a low-fat MD versus HFD or a HFD supplemented with either ursolic acid (UA) or 23-hydroxy ursolic acid (23-OHUA).

Parameter	MD		HFD		UA		23-OHUA	
	6 weeks	20 weeks	6 weeks	20 weeks	6 weeks	20 weeks	6 weeks	20 weeks
Blood glucose	86 ± 5.4	96.2 ± 6.4	111.6 ± 5.7 ^c	90.8 ± 3.9	107.5 ± 6.7 ^c	94.4 ± 5.9	103.9 ± 7.6 ^c	
Cholesterol	410 ± 7.7	490 ± 12	636 ± 42 ^c	482 ± 16	640 ± 53 ^c	532 ± 17	593 ± 54	
TG (mg/dl)	102 ± 1.1	230 ± 15 ^a	239 ± 48	210 ± 16 ^a	248 ± 37	199 ± 14 ^a	241 ± 61	
Red blood cells (10 ¹² /l)	11.1 ± 0.3	10.9 ± 0.2	9.2 ± 0.8	11.1 ± 0.2	10.8 ± 2.2	11.1 ± 0.1	9.7 ± 0.2	
White blood cells (10 ⁹ /l)	10.2 ± 1.2	9.0 ± 0.4	8.9 ± 0.8	15.8 ± 1.3	9.1 ± 0.6	10.5 ± 1.4	8.6 ± 3.3	
Lymphocytes (%)	85.3 ± 2.1	70.2 ± 3.3	62.4 ± 3.4	68.6 ± 2.6	60.8 ± 2.7	76.0 ± 2.7	58.7 ± 2.2	
Monocytes (%)	5.2 ± 1.0	4.4 ± 0.3	6.1 ± 0.6	4.6 ± 0.7	7.2 ± 1.1	4.8 ± 1.3	8.4 ± 1.1	
Platelets (10 ⁹ /l)	529 ± 67	599 ± 38	583 ± 126	460 ± 51	675 ± 86	635 ± 88	397 ± 85	

Results are shown as mean ± S.E.

^a $p < 0.05$ vs. MD;

^b $p < 0.05$ vs. HFD;

^c $p < 0.05$ vs. 6 weeks (n = 5–8).