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Ursolic Acid Protects Diabetic Mice Against Monocyte Dysfunction and Accelerated Atherosclerosis

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Abstract

Aims—Accelerated atherosclerosis is a major diabetic complication initiated by the enhanced recruitment of monocytes into the vasculature. In this study, we examined the therapeutic potential of the phytonutrients ursolic acid (UA) and resveratrol (RES) in preventing monocyte recruitment and accelerated atherosclerosis.

Methods and Results—Dietary supplementation with either RES or UA (0.2%) protected against accelerated atherosclerosis induced by streptozotocin in high-fat diet-fed LDL receptor-deficient mice. However, mice that received dietary UA for 11 weeks were significantly better protected and showed a 53% reduction in lesion formation while mice fed a RES-supplemented diet showed only a 31% reduction in lesion size. Importantly, UA was also significantly more effective in preventing the appearance of proinflammatory GR-1^{high} monocytes induced by these diabetic conditions and reducing monocyte recruitment into MCP-1-loaded Matrigel plugs implanted into these diabetic mice. Oxidatively-stressed THP-1 monocytes mimicked the behavior of blood monocytes in diabetic mice and showed enhanced responsiveness to monocyte chemoattractant protein-1 (MCP-1) without changing MCP-1 receptor (CCR2) surface expression. Pretreatment of THP-1 monocytes with RES or UA (0.3 – 10 μ M) for 15 h resulted in the dose-dependent inhibition of H₂O₂-accelerated chemotaxis in response to MCP-1, but with an IC₅₀ of 0.4 μ M, UA was 2.7-fold more potent than RES.

Conclusion—Dietary UA is a potent inhibitor of monocyte dysfunction and accelerated atherosclerosis induced by diabetes. These studies identify ursolic acid as a potential therapeutic agent for the treatment of diabetic complications, including accelerated atherosclerosis, and provide a novel mechanism for the anti-atherogenic properties of ursolic acid.

Keywords

Monocyte; diabetes; chemotaxis; atherosclerosis

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1. INTRODUCTION

The earliest events in atherosclerosis involve the recruitment of monocytes initiated by the expression of adhesion molecules by endothelial cells lining the vascular wall and the release of chemoattractant chemokines by the vasculature. Early lesions are characterized by the accumulation and prolonged persistence of monocyte-derived macrophages in the sub-endothelial space and their transformation into lipid-laden foam cells¹. Studies utilizing monocyte chemoattractant protein-1 (MCP-1) or CCR2-deficient mice demonstrated that MCP-1 and its receptor, CCR2, are intricately involved in the initiation and development of atherosclerosis². Although other chemokine/chemokine receptor pairs have recently been implicated in the development and progression of atherosclerosis, including fractalkine (CXCL13)/CXCR13 and RANTES (CCL5)/CCR5, MCP-1/CCR2 are central to all stages of atherogenesis³.

Diabetes is a metabolic disease characterized by chronic hyperglycemia and insulin resistance, which lead to macro- and microvascular complications, including atherosclerosis, peripheral vascular disease, nephropathy, retinopathy, and neuropathy⁴. Accelerated atherosclerosis is a major complication of diabetes, with diabetics having a 2–4 fold higher rate of mortality from heart-related complications than non-diabetics⁵, but the mechanisms that lead to accelerated atherosclerotic lesion formation in diabetics are not fully understood. Many metabolic diseases, including diabetes, have been linked to increased oxidative stress, the over-activation of the immune system and^{4, 6} and the dysregulation of monocyte and macrophage functions⁷. For example, monocytes isolated from diabetic patients show alterations in cell metabolism⁸, phagocytosis⁹ and cytokine production and release^{10, 11}, and similar changes in cytokine release and morphology have been reported in macrophages isolated from diabetic mice. In a mouse model of diabetic complications, we found that the diabetic condition not only increased the severity of atherosclerosis but atherosclerotic lesion size tightly correlated with increased chemotactic activity of blood monocytes *in vivo* and increased macrophage recruitment into sites of vascular and renal lesions¹². Accelerated monocyte transmigration and macrophage recruitment in turn was associated with increased intracellular thiol oxidative stress in these cells.

A number of anti-inflammatory phytonutrients have shown promise in the prevention and treatment of diabetic complications and thus may represent an affordable alternative to more traditional anti-diabetic drugs and therapies^{13, 14}. Here we examined the effect of urolic acid, an anti-inflammatory triterpenoid with well-documented anti-tumor properties¹⁵ and compared its effectiveness in preventing accelerated atherosclerosis to that of resveratrol, a phytonutrient with well-established anti-diabetic^{16, 17}, anti-atherogenic¹⁸, and cancer-preventive properties¹⁹.

RES, a stilbene found in grapes, wine, peanuts, and other plant sources²⁰, has been utilized in many studies focused on the treatment of diseases such as diabetes, atherosclerosis, and cancer²¹. RES was shown to scavenge ROS and induce antioxidant enzymes in a number of systems²². Many of RES' beneficial properties are modulated through the indirect activation of Sirtuin 1 (SIRT1)^{23, 24}, a histone deacetylase. In addition to its role in aging, SIRT1 mediates RES' ability to inhibit NF- κ B, a thiol sensitive transcription factor that controls pro-inflammatory responses^{21, 24}. This mechanism may account for the reported anti-inflammatory effects of RES in macrophages²⁵.

UA is a pentacyclic triterpenoid found in many herbs and spices like rosemary and thyme, but also in fruits including apples, cranberries, and blueberries¹⁵. UA has been primarily studied as an anti-cancer and anti-inflammatory compound. More recently, however, dietary supplementation with UA was found to improve glycemic control and lipid profiles, to

decrease lipid accumulation in the liver and increase antioxidant enzymes activity in rodent models of metabolic disease^{26, 27}. However, the effect of dietary UA on diabetic complications, particularly accelerated atherosclerosis, has not been investigated. Here we show that UA is a potent inhibitor of diabetic atherosclerosis and provide evidence that UA's anti-atherogenic activity at least to a large extent appears to involve protecting of monocytes from hyper-reactivity to MCP-1 induced by metabolic stress and accelerated cell migration.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Chemicals were purchased from Sigma-Aldrich, cell culture reagents from Gibco® and antibodies for flow cytometry were purchased from BD Biosciences unless stated otherwise.

2.2. Cell Culture

Human THP-1 monocytic cells (ATCC) were cultured in THP-1 medium (RPMI-1640 (Hyclone and Cellgro®), 10% fetal bovine serum (FBS), 2% Glutamax, 1% sodium pyruvate (Cellgro®), 1% penicillin/streptomycin (Cellgro®), 1% HEPES, 0.1% β-2-mercaptoethanol) and maintained at 37°C, 5% CO₂, and 95% humidity. All experiments used cells before they reached passage 15 and at a concentration of 0.3–0.4 × 10⁶/ml cells.

2.3. *In Vivo* Chemotaxis Assays

RES (MP Biomedicals) and UA were solubilized in dimethylsulfoxide (DMSO, <0.1% final concentration in culture medium). Cells were incubated for 15 h with either vehicle, RES or UA. RES was light-shielded to prevent isomerization²¹. Cells were washed and resuspended (1–2 × 10⁶/ml) in THP-1 medium without FBS. Subsequently, cells were treated for 2 h with either THP-1 medium (control) or THP-1 medium supplemented with 0.3 mM H₂O₂ (oxidatively stressed) and subsequently loaded into the upper wells of a 48-well modified Boyden chamber (NeuroProbe). The lower wells contained either vehicle or MCP-1 (R&D Systems). A 5 μm polyvinyl pyrrolidone-free polycarbonate membrane (NeuroProbe) was layered between the upper and lower chambers, and the chamber was incubated for 3 h at 37°C and 5% CO₂. The membrane was washed and cells removed from the upper side of the filter. Transmigrated cells were stained with Diff-Quik® Set (Dade Behring) and counted under a light microscope in five separate high power fields at 400× magnification.

2.4. CCR2 Flow Cytometry analysis

To determine cell surface expression of CCR2, THP-1 cells (1×10⁶) were washed in FACS buffer (2% FBS, 0.05% sodium azide in PBS) and F_C receptors (CD16/CD32) were blocked by incubation with human serum for 1 h. Cells were then stained for 15 min at 4°C with Alexafluor®647-conjugated mouse anti-human CCR2 antibody (CD192), washed three times with FACS buffer and analyzed using a BD FACS Calibur instrument.

2.5. 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 UTHSCSA Institutional Animal Care and Use Committee. At 11 weeks of age, mice were fed a maintenance diet (MD; AIN-93G, Bio-Serv) for 1 week to eliminate any residual phytonutrients the mice may have received with the chow diets. Mice were randomized into 4 groups: maintenance diet (MD), high fat diet (HFD; 21% fat wt/wt and 0.2%, 0.15 cholesterol wt/wt; AIN-76A, Bio-Serv), HFD + 0.2% RES or HFD + 0.2% UA. Phytonutrient-supplemented diets were prepared by Bio-Serv. All

mice designated to receive HFD were given intraperitoneal injections of streptozotocin (STZ; 50 mg/kg in citrate buffer; 50 mmol/L, pH 4.5) for 5 consecutive days to induce diabetes. Treatment diets were started one week after STZ injections and continued for a total of 11 weeks. Fasted bodyweights were assessed weekly.

2.6. Analysis of Atherosclerosis

Atherosclerosis was assessed in both the ascending and descending aorta and the aortic root. Lesion analysis was performed as described previously²⁸. Briefly, after mice were euthanatized, the right atrium was removed and hearts and aortas were perfused with PBS through the left ventricle. Hearts were embedded in OCT and frozen on dry ice. Aortas were fixed with 4% paraformaldehyde in PBS, dissected from the proximal ascending aorta to the bifurcation of the iliac artery, and all adventitial fat was removed. For *en face* analysis, aortas were opened longitudinally, pinned flat onto black paper placed over dental wax, and digitally photographed at a fixed magnification. Total aortic area and lesion areas were calculated using ImagePro Plus 6.0 (Media Cybernetics). Lesions are defined as 3 cm below the left subclavian artery and results are expressed as % lesion area of the aortic arch. As a second measure of atherosclerosis, lesions of the aortic root were analyzed. Serial sections were cut through an 800 μ m segment of the aortic root. For each mouse, 8 sections (10 μ m) separated by 80 μ m were examined. Each section was stained with Oil Red O (ORO), counterstained with hematoxylin (Vector Labs), and digitized. Lesion area was measured using ImagePro Plus 6.0 (Media Cybernetics) and expressed as millimeters squared. Macrophages were detected with anti-CD68 antibodies (Serotech)²⁸.

2.7. Blood and Urine Analysis

Mice were fasted overnight before blood was obtained by venous tail bleed periodically throughout the study or by cardiac puncture at sacrifice. Glucose was measured bimonthly using Accu-CHEK Advantage blood glucose meter. For monocyte subset analysis, blood was obtained by venous tail bleed prior to sacrifice. Plasma cholesterol and triglyceride levels were determined using enzymatic assay kits (Wako Chemicals) using blood obtained by cardiac puncture. Urine creatinine and albumin concentrations were measured by ELISA (Exocell).

2.8. In Vivo Matrigel Chemotaxis Assay

Three days before the end of the study, each mouse was injected subcutaneously into the right and left flank with growth factor-reduced Matrigel (BD Biosciences) supplemented with either vehicle or recombinant MCP-1 (300 nmol/L), respectively. The plugs were surgically removed at the time of sacrifice, dissolved in dispase (BD Biosciences), and cells were stained with calcein/AM, and counted automatically on a video-based, fluorescence cell counter (Nexcelom Bioscience) and normalized to collected plug volumes of 50 g.

2.9. Monocyte Subsets by Flow Cytometry

For the identification and quantification of monocyte subsets, whole blood was incubated in FACS buffer at 4°C, for 15 min to block F_C receptors (CD16/CD32). Red Blood Cells (RBC) were lysed with 2 ml of BD FACS Lysing Solution (BD Biosciences) and subsequently labeled with APC-labeled Gr-1 antibodies (BD Biosciences) in FACS buffer. Cells were fixed and permeabilized with Cytotfix/Cytoperm Solution (BD Biosciences) for 15 min at 4°C. Cells were subsequently washed with saponin buffer to maintain cell permeability (1% bovine serum albumin (BSA), 0.1% saponin, 0.1% sodium azide in PBS) and stained with PE-labeled anti-CD68 antibodies (Serotech) for 15 min at 4°C. All samples were washed and stored in saponin buffer prior to analysis using the BD FACSCalibur instrument.

2.10. Statistics

Data were analyzed using ANOVA (Sigma Stat 12.0). Data were tested for use of parametric or nonparametric post hoc analysis, and multiple comparisons were performed by using the Least Significant Difference method. All data are presented as mean $\pm$ SE. Results were considered statistically significant at the $P < 0.05$ level.

3. RESULTS

3.1 Dietary supplementation with ursolic acid improves metabolic profiles, kidney function, and survival of diabetic mice

To examine whether dietary supplementation of RES or UA attenuates monocyte chemotactic responses and reduces accelerated atherosclerosis in diabetic mice, we fed STZ-treated LDLR^{-/-} mice for 11 weeks a HFD or a HFD supplemented with either 0.2% RES or 0.2% UA. This mouse model of diabetic complications develops accelerated atherosclerosis in both the aortic arch and in the aortic root¹². Furthermore, blood monocytes in these diabetic mice become dysfunctional and hyper-responsive to MCP-1-induced chemotaxis. Additionally, peritoneal macrophages isolated from these same mice show increase thiol oxidative stress. Here we report that the micro- and macrovascular complications these diabetic mice develop over the course of 11 weeks of HFD feeding lead to increased mortality. The survival rate in the diabetic mice was only 40% after 11 weeks on a HFD. Importantly, survival increased to 90% in diabetic mice that received UA with their diet, but only to 70% if the diet contained RES (Fig 1). Both RES and UA significantly reduced blood glucose (BG) levels, but neither compound significantly lowered triglyceride or total cholesterol levels (Table 1). Both RES and UA, also showed significant reno-protective properties, but again UA was significantly more potent than RES in restoring kidney function ($P < 0.001$). RES reduced the urinary albumin/creatinine ratio, an indicator of kidney function, by 27% whereas UA lowered the ratio by 50% compared to diabetic mice that did not receive any phytonutrients in their diet (Table 1). Thus, dietary supplementation with either RES or UA resulted in significantly improved metabolic profiles and renal function, but UA provided significantly more pronounced beneficial effects than RES.

3.2 Dietary ursolic acid supplementation attenuates accelerated atherosclerosis in diabetic mice

To examine if dietary RES and UA affect accelerated atherosclerosis in these diabetic mice, lesion formation was analyzed in both the aortic arch and the aortic root. En face analysis of the aortic arch revealed that mice receiving dietary RES and UA showed significantly reduced atherosclerotic lesion formation in the aortic arch (Fig 2A). Again, UA was more potent and reduced lesion size by 53%, whereas RES-treated mice only showed a 31% decrease in lesions. Similar decreases in lesion size were observed in the aortic root, but the decrease in lesion size did not reach statistical significance ($P = 0.11$, Fig 2B and 2D).

The decrease in lesion size induced by either phytonutrient was accompanied by a reduction in macrophage content in the ORO-stained aortic root lesions. Macrophage infiltration into the lesions was decreased by 33% in RES-fed mice and by 31% in UA-fed (Fig 2C–D). In summary, both RES and UA protected diabetic mice against the development of atherosclerotic lesions, possibly by limiting macrophage recruitment into the lesions.

3.3 Dietary ursolic acid supplementation reduces monocyte transmigration and macrophage recruitment *in vivo*

To test whether the reduction in macrophage content in the atherosclerotic lesions of diabetic mice induced by dietary UA and RES were due to reduced monocyte recruitment,

we assessed the *in vivo* responsiveness of monocytes to MCP-1-induced monocyte chemotaxis in these diabetic mice. To this end, mice received vehicle and MCP-1-loaded Matrigel plugs in their left and right flank, respectively. As expected, monocytes in diabetic mice showed a 4.4-fold increase in chemotactic activity compared to healthy control mice (Fig 3A). However, diabetic mice that received RES or UA in their diet showed a 35% and 56% reduction, respectively, in monocyte recruitment into the subcutaneous Matrigel plugs. Importantly, reduced monocyte chemotactic activity correlated with the extent of reduction in atherosclerotic lesion formation in these mice (Fig 3B, $r^2=0.65$; $P=0.00002$). These data indicate that UA and RES may exert at least some of their anti-atherogenic properties by preventing the transformation of monocytes into a hyper-chemotactic phenotype.

3.4 Dietary ursolic acid prevents both monocytosis and the expansion of the inflammatory monocyte subset induced by diabetes

In apoE^{-/-} mice, HFD induces monocytosis and a shift in the monocytes subset distribution toward the pro-inflammatory Ly6C^{high} phenotype²⁹. To examine whether this shift occurred in our diabetic LDLR^{-/-} mice, we analyzed the level of CD68⁺ cells (monocytes) within the white blood cell population, and determined the distribution of GR-1^{high} versus GR-1^{int} and GR-1^{low} expressing monocytes within this population. GR-1 recognizes the same antigen as Ly6C. As reported for HFD-fed apoE^{-/-} mice, diabetic LDLR^{-/-} mice showed a 3.0-fold increase blood monocyte counts (Fig 4A). This increase in blood monocyte counts was due to an increase in the pro-inflammatory GR-1^{high} monocyte population although interestingly, both the GR-1^{int}, and GR-1^{low} monocyte subsets decreased by 29% and 77%, respectively (Fig 4B). As a result, the ratio of GR-1^{high} versus GR-1^{low} monocytes increased from 2 in healthy control mice (MD) to 13 in diabetic mice, indicating a dramatic shift of the monocyte population in diabetic mice toward a more inflammatory phenotype³⁰. Importantly, dietary UA completely prevented this phenotypic shift, while RES only showed partial protection (Fig 4C). Dietary supplementation with UA also completely prevented monocytosis induced by diabetic conditions, suggesting that ursolic acid may also protect the bone marrow and against the detriment effects of diabetes on hematopoiesis.

3.5 Resveratrol and ursolic acid protect human THP-1 monocytes against oxidative stress-induced hyper-responsiveness to MCP-1

In diabetic mice, RES and UA protected monocytes against hyper-reactivity to MCP-1 stimulated chemotaxis induced by diabetic conditions. Oxidative stress has been linked to the development and progression of many diabetes complications^{4, 6}, and we showed that diabetic conditions in mice promote (thiol) oxidative stress in macrophages. We therefore examined whether monocytes exposed to oxidative stress *in vitro* would mimic the hyper-chemotactic monocyte phenotype observed in diabetic mice. To this end, human THP-1 monocytes were pretreated for 2 h with H₂O₂, transferred to a micro-chemotaxis chamber, and chemotaxis was induced with MCP-1. We found that pre-exposure of monocytes to H₂O₂ accelerated MCP-1-stimulated cell migration in a dose-dependent manner, with maximal acceleration observed at 300 μ M H₂O₂ (Fig 5A). In the absence of MCP-1, pretreatment of monocytes with H₂O₂ did not alter cell migration (data not shown). To our knowledge, this is the first demonstration that oxidative stress sensitizes monocytes to MCP-1-induced chemotaxis. Our finding suggests that oxidative stress may also mediate the transformation of monocytes into the hyper-chemotactic, pro-atherogenic phenotype we observed in diabetic mice.

To examine whether we could recapitulate in cell culture the protective effects of RES and UA on accelerated monocyte migration, THP-1 monocytic cells were pretreated for 15 hours with either vehicle, UA or RES prior to a 2 h exposure of these cells to H₂O₂. Phytonutrient pretreatment resulted in a dose-dependent inhibition of the acceleration effect of H₂O₂ on

MCP-1-induced monocyte chemotaxis (Fig 5B). For all the above experiments, cells were washed prior to H₂O₂ pretreatment to avoid any direct extracellular interaction between H₂O₂ and the phytonutrients. Of note, neither RES nor UA at 10 μ M affected MCP-1-induced chemotaxis of unstressed control cells, suggesting that both phytonutrients target the cellular effects of H₂O₂ rather than MCP-1 signaling itself (not shown).

High glucose levels have been reported to increase the surface expression of CCR2, the receptor for MCP-1³¹, which may account for the increase in chemotactic activity. However, exposure of THP-1 monocytes to H₂O₂ did not alter the surface expression of CCR2, (Supplemental Figure). Furthermore, pretreating oxidatively stressed THP-1 cells with either 3 μ M RES or UA also did not alter CCR2 surface expression, yet chemotaxis of this oxidatively stressed monocyte was reduced by 81% with RES or 91% with UA. These data indicate that accelerated monocyte migration does not involve increased CCR2 receptor expression and that RES and UA appear to act downstream of CCR2 activation by MCP-1.

4. DISCUSSION

In this study, we examined whether dietary supplementation with UA or RES, two potent anti-inflammatory phytonutrients, protects against accelerated atherosclerosis associated with diabetes. We show that UA, and to a lesser extent RES, reduce atherosclerosis in the aortic root and the aortic arch. While it is possible that the reduction in blood glucose levels observed in UA and RES-fed mice may have contributed to the dramatic reduction in lesions size, the atheroprotective effect of UA and RES cannot be explained by lipid lowering as neither UA nor RES significantly reduced plasma cholesterol or triglyceride levels. Instead, our data suggests a novel anti-atherogenic mechanism for UA and RES that appears to directly target blood monocytes. Atherosclerotic lesions in UA and RES-fed mice showed significantly lower macrophage contents, suggesting that these phytonutrients either reduce the release of chemoattractants from sites of vascular injury or inhibit monocyte responses to these chemoattractants. Previously, we reported that the extent of metabolic stress directly correlates with both the macrophage content of atherosclerotic lesions as well as the responsiveness of monocytes *in vivo* to MCP-1-induced chemotaxis¹². The data we present here suggest that UA, and to a lesser extent RES, protects monocytes from metabolic stress and prevents the transformation of healthy monocytes into this hyper-chemotactic phenotype. Our studies in cultured THP-1 monocytes support this hypothesis. The mechanism by which UA and RES exert their protective effect on monocytes is not clear, but it does not appear to involve the downregulation of CCR2. Unlike RES, UA is not easily oxidized and we would predict that the underlying mechanism also does not involve scavenging of excessive ROS generated in diabetic monocytes^{10, 32}. However, UA has been reported to reduce Nox4 expression and ROS production in a human endothelial cell line³³. This finding is of particular interest as we recently identified Nox4 as a novel inducible source of intracellular hydrogen peroxide in monocytes and macrophages³⁴. Taken together our data point to a novel, monocyte-dependent mechanism through which UA (and RES) exerts its anti-atherogenic properties.

We are the first to report that UA also prevented both monocytosis induced by diabetic conditions and the phenotypical shift of blood monocytes toward a pro-inflammatory subset in diabetic mice. These data suggest that UA also affects hematopoiesis and thus appears to protect the bone marrow from the detrimental effects of diabetes. UA appears to exert its beneficial effects on multiple organ systems, which may explain the dramatically increased survival rate of diabetic mice that received the UA-supplemented diet. Previous studies by others showed that UA supplementation improves liver function in several different mouse models of acute liver injury³⁵. ICR mice rendered diabetic with STZ injections also showed improved liver function and improved lipid and glucose metabolism when on fed a HFD

supplemented with UA³⁶. Others reported improved kidney function in diabetic mice fed diets supplemented with UA^{37, 38}. The improved albumin/creatinine ratios we observed in our UA treated diabetic mice confirm these findings.

UA's bioavailability and pharmacokinetics are not well characterized. Together with the limited information available on the level of UA in different food sources make it difficult to accurately assess the therapeutic potential of UA in the human diet or its usefulness as a dietary supplement. The lack of chromophores in UA and its resistance to oxidation make it difficult to detect and accurately quantify UA in plasma and determine UA's bioavailability. Based on a 0.2% UA in the HFD and an average intake of 3 g of food per day we estimated the daily intake of UA for our mice to be approximately 300 mg/kg bodyweight. Using the body surface area normalization method to convert the mouse dosage to an approximate dosage for humans³⁹, this dose translates into a dose of 24 mg/kg in humans. To put this in perspective, to achieve this dose a 70 kg person would need to consume 1.7 g/day of UA which amounts to approximately 34 apples⁴⁰ or 15 servings (100 g) of cranberries⁴¹. While dietary sources may not be able to provide these doses of UA, it may be possible to achieve these doses through supplementation⁴¹. UA-containing extracts are commercially available, but the purity, dosage, and effective bioavailability of these products have not been validated, as is the case with most dietary supplements. Currently, there are no detailed studies that address UA's toxicity, but, oleanolic acid, a structural isomer of UA, is considered non-toxic⁴². However, it is quite possible that UA may act synergistically with other micronutrients in our diet, and significantly lower doses of UA than those used in our studies may be needed to achieve atheroprotection. In summary, we identified UA as a novel anti-atherogenic phytonutrient and provide evidence that UA's ability to protect blood monocytes from diabetes-induced dysfunction contributes to its athero-protective properties.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Alb	Albumin
BG	Blood Glucose
Cre	Creatinine
HFD	High-Fat Diet
LDL	Low-density Lipoprotein
MD	Maintenance Diet
ORO	Oil Red O
ROS	Reactive oxygen species
RES	Resveratrol
STZ	Streptozotocin
TC	Total Cholesterol

TG	Triglycerides
UA	Ursolic Acid

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Figure 1. UA and RES increase survival of diabetic mice fed a HFD

Survival curve showing study survival of diabetic mice fed a HFD (open circles, solid line), or a HFD with either 0.2% RES (gray triangles, dotted line) or 0.2% UA (closed circles, dashed line). Results are expressed as percent survival.

Figure 2. UA and RES attenuate atherosclerotic lesion formation in diabetic mice

A) Lesion area in aortic arch was analyzed by *en face* analysis as described under *Materials and Methods* (2.6). Results are expressed as % lesion area per entire lumen area $\pm$ SE. *: versus HFD, $P < 0.001$; #: versus RES, $P = 0.01$; $n = 5-9$. **B)** Lesion area in the aortic root was determined in 10 ORO-stained sections of the aortic root separated by 80 μ m. Results are expressed in $\text{mm}^2 \pm$ SE. **: versus HFD, $P = 0.016$; $n = 5-7$. ANOVA showed no statistically significant difference between the three treatment groups ($P = 0.11$). However, posthoc analysis (Holm-Sidak method) revealed a significant decrease in lesion size ($P = 0.016$) in the group that received UA compared with the unsupplemented diabetic control group (HFD). **C)** Macrophage content in aortic root lesions. Macrophages were visualized by staining 10 adjacent sections of the aortic root separated by 80 μ m with antibodies directed against CD68. *: versus HFD+STZ, $P = 0.016$ (for RES), $P = 0.011$ (for UA); $n = 4-6$. **D)** Representative images depicting ORO staining (red) and CD68 immunohistochemistry (brown) of aortic root sections. Magnification: 40 $\times$.

Figure 3. UA and RES reduce monocyte recruitment *in vivo*

A) Quantitation monocyte-derived macrophages recruited into vehicle (–) or MCP-1-loaded (+) Matrigel plugs implanted into the mice as described in *Materials and Methods* (2.8). MD (hashed bars), STZ-HFD (white bars), RES (grey bars), UA (black bars). Results are expressed as macrophages/plug $\pm$ SE. *: versus MD, $P < 0.001$; #: versus HFD, $P = 0.008$ (RES); $P < 0.001$ (UA); UA versus RES $P = 0.052$; $n = 5-9$. No significant differences were observed between groups of vehicle-loaded plugs. **B)** Linear correlation shown between % lesion area of aortic arch and macrophage recruitment into Matrigel plugs, HFD (open circles), RES (grey triangles), UA (closed circles); $r^2 = 0.065$, $P = 0.00002$, $n = 5-9$.

Figure 4. UA and RES reduced monocytois, prevented accumulation of inflammatory monocyte subset

A) Blood monocytes were analyzed by flow cytometry using an anti-CD68 PE-conjugated antibody to identify monocytes and an APC-labeled Gr-1 antibodies antibody to distinguish monocyte subsets in blood from diabetic mice fed either a MD (hashed bar), a HFD (open bar) or a HFD supplemented with either 0.2% RES (gray bar) or 0.2% UA (black bar). Results are expressed as %CD68-positive cells of total white blood cells $\pm$ SE. *: versus MD, $P < 0.001$; #: versus HFD, $P < 0.001$, $n = 4-9$. **B)** Monocyte subsets: White blood cells (WBC) were stained with antibodies directed against Gr-1 and the monocyte marker CD68 and analyzed by flow cytometry. Monocyte subsets (CD68 positive cells) were distinguished based on their level of GR-1 surface expression: GR-1^{high} (black bars), GR-1^{int} (grey bars), and GR-1^{low} (open bars). *: versus MD, $P < 0.001$; #: versus HFD, $P < 0.001$, + versus RES $P < 0.001$ $n = 3-9$. **C)** Ratios of classical or inflammatory GR-1^{High} to nonclassical Gr-1^{Low} monocytes were calculated based on the data obtained in panel B. Results shown are means $\pm$ SE. *: versus MD, $P < 0.001$; #: versus HFD, $P < 0.001$, +: versus RES, $P = 0.003$; $n = 3-9$.

Figure 5. UA and RES attenuate H₂O₂-induced acceleration of monocyte chemotaxis in response to MCP-1

A) THP-1 cells were pretreated with 0.1 – 3 mM H₂O₂ for 2 h, and then stimulated with 2 nM MCP-1 for 3 hours. Migrated cells were counted in 4–5 high power fields (HPF) per well; four wells per condition were tested in each experiment. Data are normalized to MCP-1-stimulated monocytes that did not receive H₂O₂ (0). Results are shown as mean from 8 independent experiments $\pm$ SE (1.0 = 60 ± 17 cells/hpf). *: versus Control (no H₂O₂), $P < 0.033$; #: versus 0.3 mM H₂O₂, $P = 0.002$. **B)** THP-1 cells were pretreated for 15 h with either UA (black circle) or RES (grey triangle) prior to treatment with 0.3 mM H₂O₂. Monocyte chemotaxis was quantified as in panel A. RES (grey triangles), UA (closed circles). Data were normalized to the accelerating effect of H₂O₂ on chemotaxis (“100%”), i.e. values obtained for H₂O₂-pretreated THP-1 monocytes stimulated with MCP-1 minus values obtained for non-pretreated THP-1 monocytes stimulated with MCP-1 (“0%”; dotted line); Values greater than 0% indicate accelerated chemotaxis, values below 0% indicate inhibition of MCP-1-induced chemotaxis. Results are from 4 independent experiments are shown as mean $\pm$ SE. *: versus 100% accelerated migration, $P < 0.001$ (3, 10 μ M RES); #: $P = 0.011$, $n = 4$.

Table 1

Body weight, metabolic profile and kidney function in healthy and diabetic mice fed a RES or UA-supplemented diet

Body weights, fasted plasma lipid, blood glucose levels and urinary albumin and creatinine levels were determined at the time of sacrifice as described under *Materials and Methods*. Results are expressed as means $\pm$ SE. *, **, versus HFD,

Mice	Weight (g)	BG (mg/dl)	TC (mg/dl)	TG (mg/dl)	Alb/Cre Ratio (ug/mg)
MD	23.3 $\pm$ 0.7	84.5 $\pm$ 8.3	420 $\pm$ 23	101 $\pm$ 1.9	25.6 $\pm$ 1.8
HFD	15.5 $\pm$ 0.5	320 $\pm$ 46	737 $\pm$ 34	345 $\pm$ 61	832 $\pm$ 35
RES	18.3 $\pm$ 0.8 **	142 $\pm$ 33 *	853 $\pm$ 3.2	301 $\pm$ 84	605 $\pm$ 25 **
UA	19.5 $\pm$ 0.6 *	170 $\pm$ 20 *	636 $\pm$ 4.2	328 $\pm$ 83	412 $\pm$ 6.4 *

* $P < 0.01$;

** $P = 0.05$.